REPORT

DRAFT FINAL REPORT

Task 97-55: Transition of Assay Techniques Developed by U.S. Army Medical Research Institute of Chemical Defense to the Medical Research and Evaluation Facility

To

U.S. Army Medical Research and Development Command

July, 1999

DRAFT FINAL REPORT

Contract No. DAMD17-89-C-9050
A Medical Research and Evaluation Facility (MREF) and Studies
Supporting the Medical Chemical Defense Program

on

TASK 97-55
Transition of Assay Techniques Developed by US Army Medical Research
Institute of Chemical Defense to the Medical Research and Evaluation Facility

to

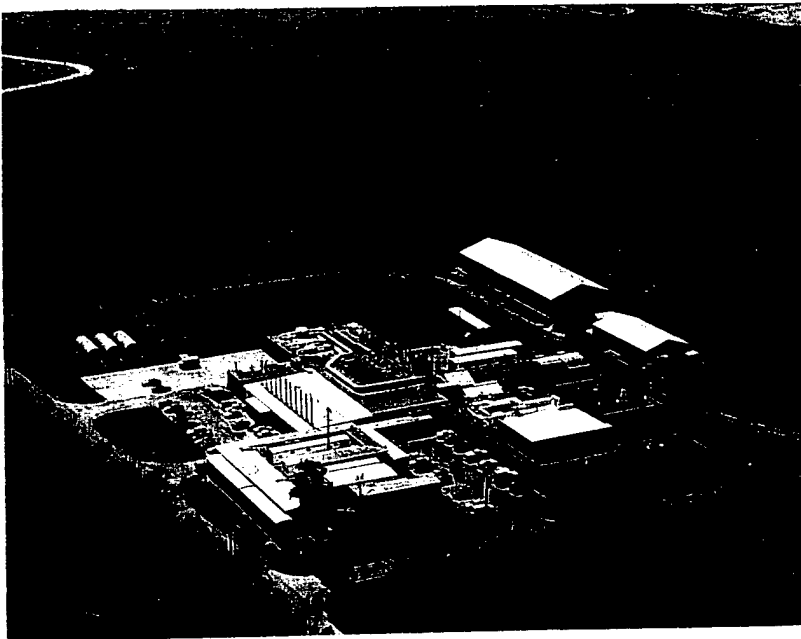## U.S. ARMY MEDICAL RESEARCH
INSTITUTE OF CHEMICAL DEFENSE

July, 1999

by

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EXECUTIVE SUMMARY

"TRANSITION OF ASSAY TECHNIQUES DEVELOPED BY USAMRICD TO THE MREF"

INTRODUCTION

"The Transition of Assay Techniques Developed by USAMRICD to the MREF" was performed under Contract DAMD 17-89-C-9050. The task is composed of the validation of three of the methods reported in Technical Bulletin (TB MED 296), entitled "Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman, GF, and Cyanide." The three selected methods are:

- Chapter 2, Verification of Sulfur Mustard Exposure – Measuring Thiodiglycol in Urine by Gas Chromatograph/Mass Spectrometer
- Chapter 3, Verification of Nerve Agent Exposure – Monitoring Blood Cholinesterase Activity with the Test-Mate™ OP Kit
- Chapter 4, Verification of Nerve Agent Exposure – Measuring Alkylmethylphosphonic Acids in Urine By Gas Chromatograph/Mass Spectrometer

The following report documents the work conducted to transfer the methods to Battelle and the results of Battelle's method validation process. A brief summary of each method is provided below.

VALIDATION OF A GAS CHROMATOGRAPH/MASS SPECTROMETER METHOD FOR VERIFICATION OF EXPOSURE TO MUSTARD

The procedure for analysis of the metabolite of sulfur mustard, thiodiglycol, described in TB MED 296, May 1996, did not yield data with sufficient precision and accuracy for routine use in our laboratory. After discussing this with the authors, a draft revised method was sent to Battelle in September of 1998. However, Battelle found it necessary to make additional changes, including changing the extraction solvent from ethyl acetate to benzene and adding a heating step during the derivatization of the thiodiglycol, to get reliable results. The Battelle modified

method was followed during the validation process. The linear range for the method was found to be 5-250 ng/mL thiodiglycol, with a detection limit of approximately 5 ng/mL and a quantitation limit of 20-25 ng/mL. From our data, the accuracy of the method is dependent upon the matching of the matrix used to generate the standard curve with that of the unknown samples. At concentration levels of less than 25-50 ng/mL, either the pre-exposure urine of the patient or the method of standard additions (over-spiking of post-exposure urine) <u>must</u> be used for accurate calibration. Above 50 ng/mL, water may be used for calibration; however, pooled urine or pre-exposure urine of the patient remains preferable.

VALIDATION OF THE TEST-MATE KIT AS A METHOD OF MONITORING BLOOD CHOLINESTERASE LEVELS

The Test-Mate™ OP Kit, as referenced in TB MED 296, was discontinued by the manufacturer (EQM Research) prior to the start of the task. It was replaced with the Test-mate ChE Cholinesterase Test System, which is reported to be more accurate and the results more reproducible.

As with the original Test-Mate™ kit and other standard methods of detecting cholinesterase inhibition (centrifugal analyzers), the new kit is based upon the chemistry of the Ellman method. As such, it contains the same limitations – a baseline cholinesterase level must be determined prior to exposure; results are relative only, absolute results from one method cannot be compared with the actual results from other methods. The system does appear to be a quick, reproducible method of determining cholinesterase depression. The kit is portable and operators with minimal training easily obtain the results in 3-4 minutes. The amount of enzyme depression is linear, as determined by the analysis of partially inhibited blood samples.

VALIDATION OF A GAS CHROMATOGRAPH/MASS SPECTROMETER METHOD FOR VERIFICATION OF NERVE AGENT EXPOSURE

The method of detecting phosphonic acids, nerve agent metabolites, in urine was validated as written in TD MED 296. Added emphasis must, however, be placed on the necessity of drying the potassium carbonate catalyst used in the method. It is also important that the water be removed from the C18 extraction cartridges before eluting the analytes with methanol since the derivatizing agent, pentafluorobenzyl bromide (PFBBR), decomposes readily in water.

The linear range was approximately 5-200 ng/mL for all three phosphonic acids – isopropyl methylphosphonic acid (IMPA), pinacolyl methylphosphonic acid (PMPA) and cyclohexyl methylphosphonic acid (CMPA). The analytes could be detected at concentrations as low as 0.3 ng/mL; however, the quantitation limit is about 20 ng/mL. In most cases, pooled urine or pre-exposure urine of the patient is the preferred matrix for generation of regression lines. At low concentration levels, a water matrix actually yielded more accurate results for IMPA.

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APPENDIX B

RECOMMENDED CHANGES TO
CHAPTER 2, TECHNICAL BULLETIN, TB MED 296, "ASSAY TECHNIQUES FOR DETECTION OF EXPOSURE TO SULFUR MUSTARD, CHOLINESTERASE INHIBITORS, SARIN, SOMAN, GF AND CYANIDE", MAY 1996.

APPENDIX C

METHOD NO. 18/CHEMISTRY
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TASK 97-55
VALIDATION OF A GAS CHROMATOGRAPH/MASS SPECTROMETER METHOD FOR VERIFICATION OF EXPOSURE TO MUSTARD

1.0 INTRODUCTION

Battelle's Medical Research and Evaluation Facility (MREF) was requested by the United States Army Medical Research Institute of Chemical Defense (USAMRICD) to validate a gas chromatograph/mass spectrometer method for the detection of a metabolite of sulfur mustard (HD), thiodiglycol, in human urine. The work was done under Contract DAMD 17-89-C-9050.

The method has been reported in Technical Bulletin (TB MED 296), Chapter 2, "Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman, GF, and Cyanide", Headquarters, Department of the Army, May 1996 (Appendix A). There have been numerous papers published which deal with the metabolites of sulfur mustard, thiodiglycol (TDG) being the compound most commonly mentioned.[1] Analytical methods for quantitation of TDG are generally complicated by the reactivity of the analyte.[1] As with phosphonic acids, the metabolites of nerve agents, it is crucial to the military to be able to identify and quantitate the agent involved in an exposure for treatment purposes. The method that is decided upon must then be shown to be reproducible on a routine basis by other laboratories.

Several series of analyses of TDG in water, urine and ethyl acetate matrices were performed according to the method as written in TB MED 296. None of the data obtained by Battelle in this testing met with our expectations for a reliable method. Battelle subsequently contacted the author of the method to discuss the difficulties encountered. Personnel at USAMRICD confirmed that they were obtaining results similar to those obtained at Battelle using the published method. They were working to improve the method and transmitted a copy of recommended changes on September 22, 1998 (Appendix B). Their message summarized the problems and suggested solutions, such as the elimination of thiodipropanol (TDP) and the inclusion of pyridine. The main areas of concern identified were:

- Loss of TDG during evaporation of the urine to dryness
- Evaporation of the derivatized TDG in the final concentration step
- Possible decomposition of the derivatized TDG in the acid environment which inevitably results when using excessive amounts of heptafluorobutyric anhydride for derivatization
- Possible side reactions in the injection port in the presence of heptafluorobutyric acid

With this information it was decided that Battelle would conduct the task in two phases, a short development stage using the recommendations from the draft method contained in Appendix B, and a method validation phase. A new method was written based on the data obtained during the development process (Appendix C), as documented in this report.

2.0 PRELIMINARY INVESTIGATION AND DISCUSSION

The initial concern was the extraction of thiodiglycol from aqueous samples. Thiodiglycol is highly soluble in water. There are a limited number of solvents that are not water soluble for which the TDG extraction coefficient is greater than that of water and that possess suitable chromatographic properties. The approach used in the original TB MED 296 method was to evaporate aqueous and urine samples to dryness at a temperature of 90 C under a stream of nitrogen after an enzyme digestion and pH adjustment and then redissolve the residue with ethyl acetate. Following derivatization, the sample was taken to dryness, again at 90 C under a nitrogen flow, and the residue dissolved in 100 µL ethyl acetate. In both the findings of USAMRICD and Battelle, the final drying step after derivatization resulted in a marked loss of TDG. To estimate the loss, solutions containing approximately 500 ng/mL derivatized TDG were analyzed before and after heating. Table 1 lists the approximate relationships between TDG depletion, temperature, and time required for the final drying step.

Table 1. Reduction in TDG with Temperature and Drying Time

Temperature	Reduction in TDG Response	Time
20 C	30%	3 hr
60 C	40%	2 hr
90 C	80 %	15 min

After review of these data, it was decided that the heating of the samples to dryness following derivatization was not a suitable way to concentrate the TDG. Several original parameters were also evaluated: sample pH adjustment and drying reagents besides molecular sieves. Adjusting pH to precipitate biological materials or stabilize TDG was found not to be necessary, and using anhydrous sodium sulfate was superior to using molecular sieves for drying of the solvent. The procedural change that may have produced the greatest improvement was replacing ethyl acetate with benzene in the method. Although TDG is soluble in ethyl acetate, it is not the solvent of choice from a gas chromatography standpoint. Due to ethyl acetate's relatively high boiling point, the initial column temperature must be kept high to prevent column flooding, which results in split chromatographic peaks. Ethyl acetate not only combines with a considerable amount of water, but also forms an azeotrope with it (6.1 percent w/w); therefore, after the extraction process it is difficult, if not impossible, to remove all the water prior to derivatization, which must be carried out in a water-free solvent. Therefore, the selection of a new solvent for extraction and derivatization was considered. Based upon previous experience, benzene was our first choice. For these reasons and recommendations [2,3] from Pierce Chemical, the manufacturer of the derivatizing agent, the extraction solvent was changed from ethyl acetate to benzene. The solubility of benzene in water is low (0.19 percent w/w at 24 C). Derivatization was carried out at 50 C for 15 min, as recommended by Pierce.

In the USAMRICD modified method, only a portion of the initial sample extract (250 µL of 1 mL) was derivatized, and only a portion of the resulting derivatized solution (50 µL of 313 µL) was added to the cleanup cartridge. The 50 µL of derivatizated solution added to the cartridge was further diluted by rinsing the column with 450 µL of solvent. The net effect is that the amount of sample reaching the final step of analysis by

gas chromatography/mass spectrometry (GC/MS) has been reduced by a factor of approximately 15,000. The validated method was simplified by carrying the entire extract through the full process. By using the entire sample, the sensitivity of the method also is improved.

Organic bases, e.g., pyridine, are commonly used in derivatization reactions involving anhydrides to neutralize excess acids formed during the reaction. The excess acid, if not neutralized, contributes to sample instability and damage to the chromatographic column. The initial 3 µL of pyridine, followed by cleanup of the sample with a silica cartridge (conditioned with benzene), was sufficient to remove the acids. The additional 30 µL of moist pyridine used in the modified method was found to be unnecessary. The extra base caused rapid decomposition of the chromatographic column and a marked decrease in the sensitivity of the instrument. Mass spectrometer source and column performance was decreased to unacceptable levels after only five to six sample injections.

3.0 EXPERIMENTAL PROCEDURES

The TDG analysis method is summarized in the following paragraphs, with a detailed description of the experimental procedures provided in MREF Method No. 18-01/Chemistry located in Appendix C. TDG is the only metabolite of HD determined in this method. D_8-TDG is used as an internal standard. Thiodiglycol was purchased from Aldrich Chemical Co. D_8-TDG was synthesized by Dr. Allison Fentiman of Battelle.

Approximately one liter of urine was collected over a one to two day period from each of nine volunteers for use in this study. After approximately one liter of urine had been received from a particular donor, the jar was thoroughly mixed and 10-12 mL portions were aliquoted into labeled, plastic screw-cap centrifuge tubes. The centrifuge tubes were placed in test tube racks, which were enclosed in a plastic bag and stored in a -20 ± 10 C freezer. Samples were removed and thawed only as needed for testing.

A 1 µg/mL TDG solution and a 5 µg/mL d_8-TDG working standard were prepared in methanol. Using these stock solutions, a series of aqueous and urine standards were prepared for analysis by spiking eight microcentrifuge tubes containing

1-mL of each matrix with 0, 2.5, 5, 10, 25, 50, 100, and 250 μL of the 1μg/mL TDG working standard. Twenty μL of the 5 μg/mL d_8-TDG working standard was added to each of these eight microcentrifuge tubes so that the concentration of the internal standard would be approximately at the mid-concentration range of the analytes, 100 ng/mL. By carrying out the analysis with various matrices, matrix-related biases in the data should become evident.

After vortexing each microcentrifuge tube, the tubes were placed in a heating block at 70-75 C and the solutions were allowed to evaporate down to a volume of approximately 80 μL under a stream of nitrogen. To prevent loss of TDG and d_8-TDG, it is important that the temperature of the heating block not be above 75 C or the tubes allowed to go to dryness. The time for this concentration step is matrix dependent and typically takes between 60 and 90 min to complete.

Once the samples were concentrated to a semi-solid residue, each tube received 500 μL of benzene. After vortexing, approximately 200 mg of finely ground anhydrous sodium sulfate was added. This was a sufficient quantity to provide free-flowing powder in the sample. It is important that the sodium sulfate be in powder form and not a coarse granular form to maximize absorption of water. If the particle size was too small, however, there was difficulty in removing the benzene layer free of sodium sulfate fines. Also, small amounts of sodium sulfate on the lip of the microcentrifuge tubes can prevent the tubes from sealing properly. Each tube was again vortexed, not only to increase the adsorption of water, but also to prevent the clumping of the sodium sulfate. The tubes were placed on an automatic vortexer for 10 min and then centrifuged for 1 min. The extracts were transferred to labeled microcentrifuge tubes. An additional 500 μL of benzene was added to the initial tubes containing the residue and the extraction process was repeated. The second extract was combined with the first and the tubes containing residues were discarded. Approximately 100 mg of additional sodium sulfate was added to the tubes containing the two 500-μL benzene extracts. The tubes were votexed briefly and then centrifuged for approximately 1 min. The entire extracts were transferred to clean microcentrifuge tubes.

To each benzene extract was added 30 μL of heptafluorobutyric anhydride (HFBA) and 3 μL of dried pyridine (pyridine stored over molecular sieves). Pyridine

adsorbs excess heptafluorobutyric acid and drives the reaction to completion. The tubes were vortexed and placed in a heating block at 50 C for 15 min.

An appropriate number of silica cartridges (one for each microcentrifuge tube) were conditioned with 1 mL of benzene. The solution in each microcentrifuge tube was added to a cartridge and eluted into a labeled autosampler vial for analysis by GC/MS. The silica removes acidic byproducts from the mixture to prevent breakdown of the column phase and possible decomposition of the derivatized analytes.

A Micromass Quattro II mass spectrometer set for selected ion monitoring (SIM) in the 70 eV electron ionization mode was used to analyze all samples. The ions used for quantitation were 241 m/e for TDG and 245 m/e for d_8-TDG. An additional ion at 300 m/e was monitored for confirmation of TDG and the 307 m/e ion was used for d_8-TDG. Calculations also were performed on a portion of the data using the 300 m/e and 307 m/e ions for quantitation. This was done to compare reproducibility of the analysis and interferences between the two ion pairs.

Samples were chromatographed using a Fisions/Thermoquest 8000 Series II equipped with an AS 800 autosampler using the following conditions:

Column   HP-5MS, 15 m, with a 0.25-mm internal diameter and 0.25 μm film

               thickness

Column Temperatures
               60 C for 2 min,
               60 C to 110 C at a rate of 40 C/min
               110 C to 125 C at a rate of 3 C/min
               125 C to 300 C at a rate of 49 C/min
               300 C for 2 min

Typical Retention Times

       d_8-TDG   6.01 min

       TDG   6.08 min

Peak areas were used for all calculations. Full scan spectra for the TDG and d_8-TDG are shown in Figures 1 and 2. Extracted ion chromatograms are shown in Figure 3.

4.0 VALIDATION

The urine of seven donors was arbitrarily chosen from the nine that were originally collected. A full set of eight concentration levels of TDG with an internal standard was prepared in water and in the seven urine samples. Thus, for each set a total of 64 samples (8 concentration levels x 8 matrices) were carried through the method described above. This procedure was repeated a total of five times on non-consecutive days. Individual calibration curves were generated for each of the eight matrices and the concentrations of TDG calculated using the regression lines which had been determined. In addition, concentration levels of the TDG were re-calculated for all samples in each set using three different methods. The first method used the water standards for calibration, the second, urine donor #2 standards for calibration, and the third, standards from urine donor #4.

The differences between the calculated TDG concentrations and the spiked TDG concentrations were tabulated for all analyses. For this study, the quantitation limit was defined as the concentration at which the average percent difference and the percent RSD of the difference between spiked and actual analyte concentrations for the five day testing period was no more than 15 percent. The detection limit is normally defined as the point at which the analyte signal to noise ratio is approximately 3:1. This analysis was found to be highly matrix dependent. Although the signal to noise ratio for the lowest 2.5 ng/mL standard was greater than 3:1, the detection limit was considered to be the point at which the TDG peak can consistently be quantitated above background interferences.

5.0 RESULTS AND DISCUSSION

Linearity

Examples of regression lines for each analyte in both water and urine matrices are shown in Figures 4-11. A second order quadratic was found to fit the data better than a first order regression. This is primarily due to the wide concentration range of the standards. As listed in the text for each figure, the correlation coefficients are all greater than 0.99. The average values of the correlation coefficients over the five trials are listed in Table 2, along with percent RSD values. The water values are an average of the one

water sample over 5 trials. Urine values are an average of 35 values – 7 urine samples over 5 trials.

Table 2. Correlation Coefficients Over Five Trials

	Correlation Coefficient	%RSD
Water	0.997	0.2 %
Urine	0.999	0.2 %

A correlation coefficient of 0.99 or greater for regression lines is considered acceptable for most applications. This value should also be obtainable in analyses on different days. From the data generated, a second order regression model fits the data in the range of 2.5-250 ng/mL. A percent RSD of less than 0.2 indicates acceptable reproducibility of the linearity over time.

Quantitation Limit

Table 3 on page 17 contains the calculated analyte concentrations in water and in each of the seven urine samples using the regression lines generated for each individual matrix. The average of the 5 trials, the percent difference between spiked and calculated concentrations, the standard deviation and percent RSD for the spiked and calculated differences also have been included. Table 4 is a summary of the urine percent RSD values presented in Table 3. A quantitation limit of 20 ng/mL was based upon analysis of the data in Table 4. The percent difference between the calculated and spiked TDG concentrations in urine does not exceed 15 percent until the TDG falls below 10 ng/mL; however, the percent RSD over the 5 trials is 15 percent at approximately 20 ng/mL. Using the same criteria, the quantitation limit in water is slightly higher, 25-30 ng/mL, as can be seen from the data at the top of Table 3.

Matrix Interferences

In an actual exposure situation, it may not be possible to obtain pre-exposure urine of the victims for standards preparation. Water or pooled urine may be the only matrices readily available. For this reason, the analyte concentrations in all samples of each set were recalculated using either water, urine donor #2 or urine donor #4 samples

for the calibration standards. The results are summarized in Table 5. From this data, it can be seen that below concentrations of 25-50 ng/mL, the sample and standard matrices must be the same to obtain reliable results. The method of standard additions, i.e., overspiking of post-exposure urine with the analyte of interest, is also acceptable.

Detection Limit

Using the Masslynx software of the mass spectrometer, the signal to noise ratios for the 2.5 ng/mL TDG standards in water and urine were found to be in the range of 30:1 to 1000:1. While this range is obviously much larger than the 3:1 ratio normally used in determining detection limits, there is too large a variation in calculated TDG concentrations at even the 2.5 ng/mL level to consider a detection level any lower than this value. As shown in Table 2, matrix interferences make it difficult to distinguish reliably between concentrations of 2.5 and 5.0 ng/mL TDG. A detection limit of 5 ng/mL is considered a more reasonable estimate of a detection limit.

Alternative Quantitation Ions

The raw data obtained in Trial 1 was reanalyzed using the alternative quantitation ions 300/307 m/e. This was done to determine whether either set of ions results in a better correlation between spiked and calculated TDG values. The absolute area counts of the 241/245 m/e ions were already known to be approximately 3-5 times greater than the 300/307 m/e ions for a given concentration level. It is for this reason that the 241/245 m/e ions were chosen initially. Table 6 contains a summary of the TDG concentrations calculated using the two different sets of quantitation ions. Each matrix was used to generate its own regression line. Tables 7-9 contain values obtained using water, urine donor #2 urine and urine donor #4 urine to generate regression lines.

When each matrix is used to generate a regression line and the TDG concentrations are calculated, there appears to be little improvement when using the 300/307 m/e ions. However, if water, urine donor #2 urine or urine donor #4 urine is used to generate regression lines, there is some improvement when using the 300/307 m/e ions at the lower concentration levels. This implies that there is a urine dependent interference and, as expected, it is more noticeable at lower concentrations.

6.0 CONCLUSION

The validated method is an acceptable means of determining TDG concentrations in human urine in an approximate concentration range of 5-250 ng/mL. TDG can reliably be detected at 5 ng/mL and quantitated between 20-25 ng/mL. Pooled urine or water may be used to generate regression lines for analyses in the higher concentration ranges (>50 ng/mL). However, at the lower concentration levels it is imperative that pre-exposure urine of the patient be used for calibration or that the method of standard additions, over-spiking of the post exposure urine, be used.

It is important that the urine samples not be heated excessively in the pre-extraction step and that the solvent used for derivatization be completely dry. It is recommended that HFBA be purchased in 1-mL sealed ampoules rather than in a screw-cap bottle since moisture will readily decompose the reagent.

7.0 REFERENCES

1. Technical Bulletin (TB MED 296), "Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman, GF, and Cyanide", Headquarters, Department of the Army, May 1996.

2. Instructions, Acylation Derivatization Reagents (0765), Pierce Chemical, Rockford, IL.

3. Knapp, D.R. *Handbook of Derivatization Reactions,* John Wiley and Sons, Inc., N.Y. (1979).
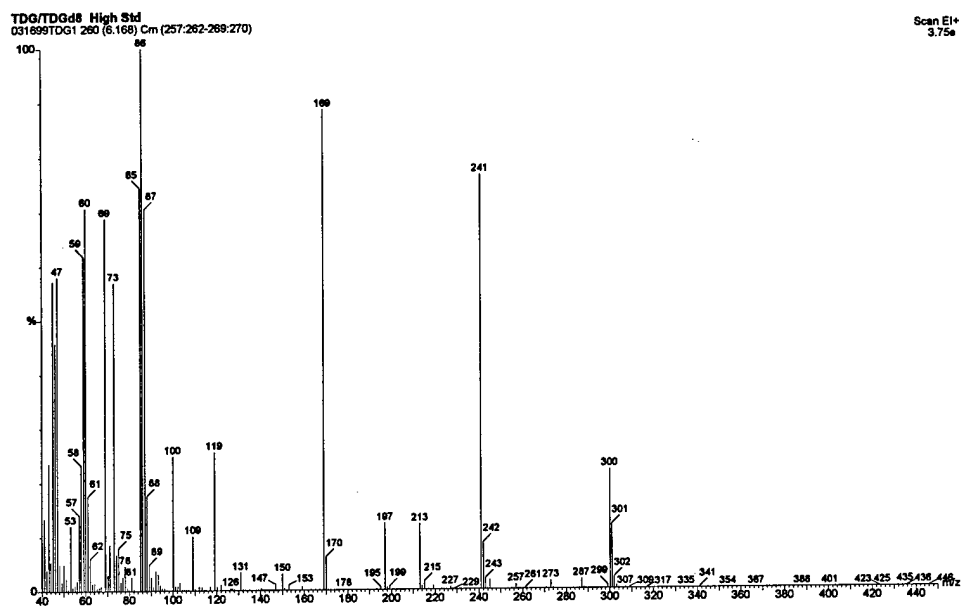
Figure 1: Spectrum of HFBA Derivative of TDG
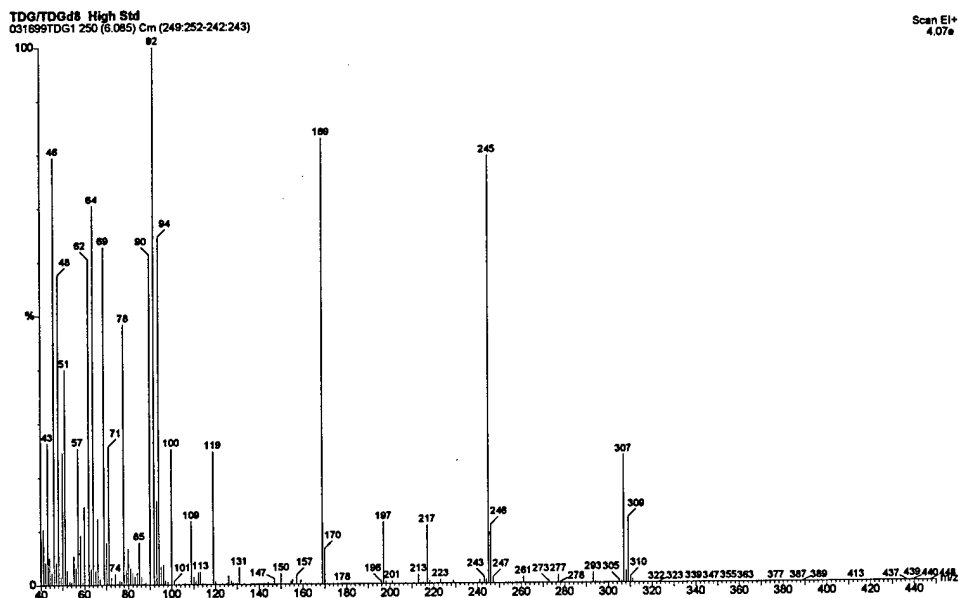
Figure 2: Spectrum of HFBA Derivative of d8-TDG
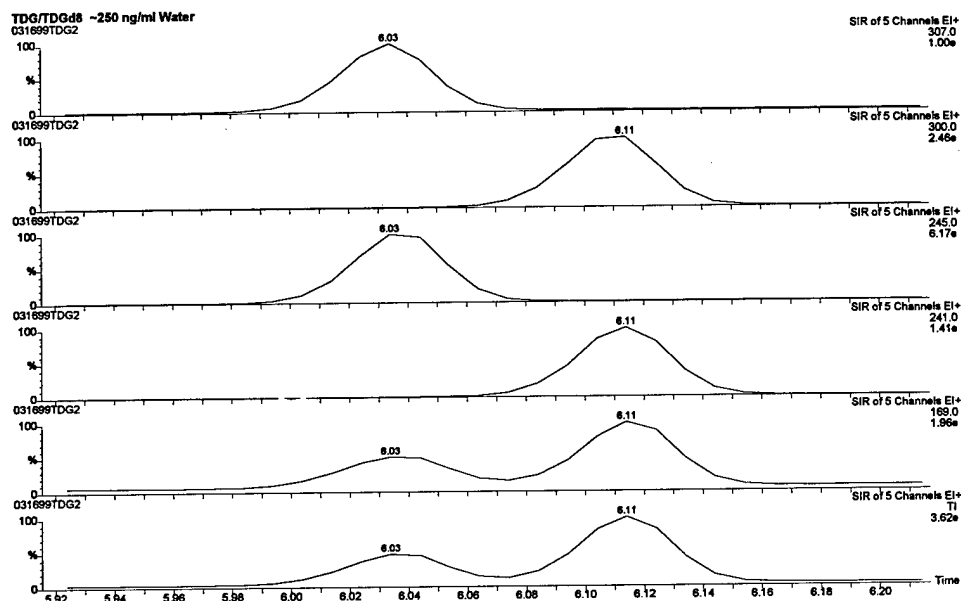
Figure 3: SIM for TDG and d₈-TDG
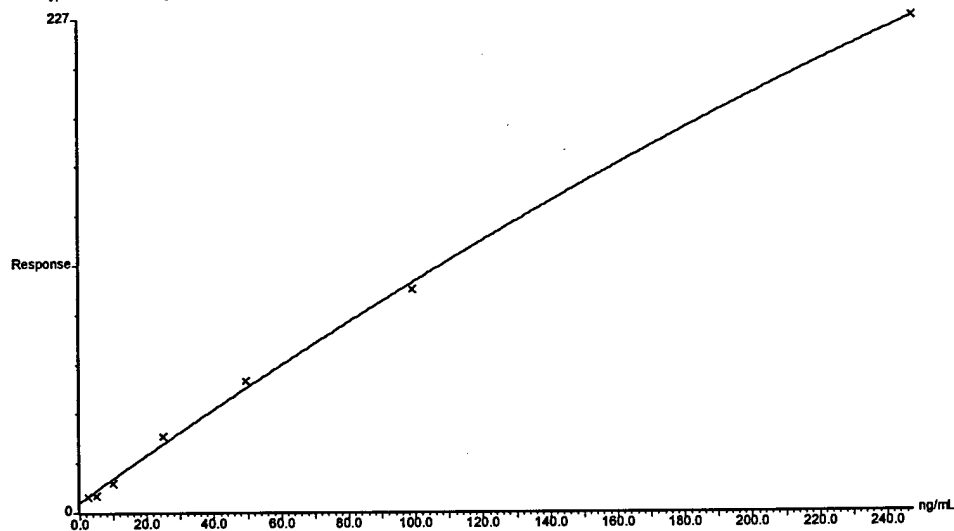
Figure 4: Regression Line for TDG in Water

Compound 1 name: HFBA Der. of TDG
Coefficient of Determination: 0.999732
Calibration curve: -7.19850e-5 * x^2 + 1.00488 * x + 6.38660
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: Null, Axis trans: None
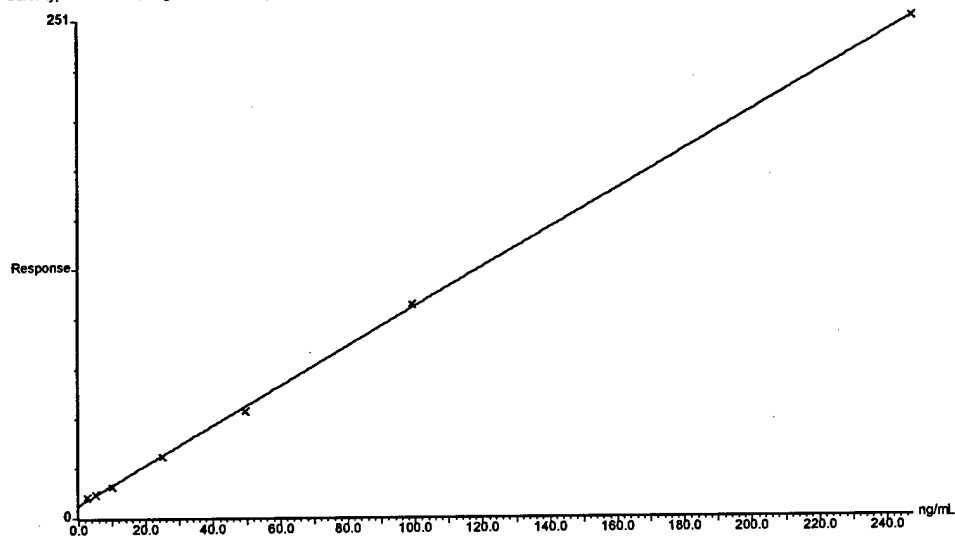
Figure 5: Regression Line for TDG in Urine Donor #1

Compound 1 name: HFBA Der. of TDG
Coefficient of Determination: 0.999413
Calibration curve: -0.000609162 * x^2 + 1.10323 * x + 26.2128
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: Null, Axis trans: None
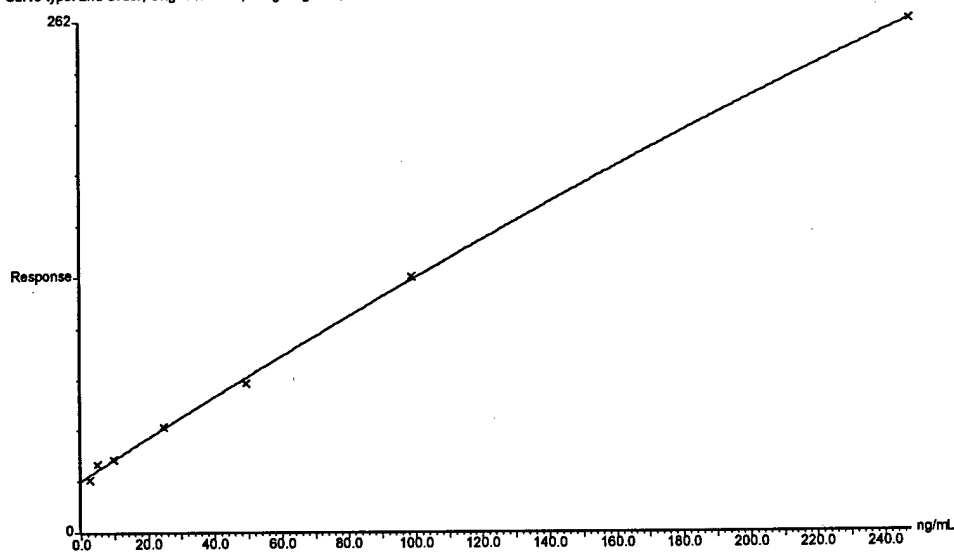
Figure 6: Regression Line for TDG in Urine Donor #2

Compound 1 name: HFBA Der. of TDG
Coefficient of Determination: 0.995506
Calibration curve: -0.00125298 * x^2 + 1.31260 * x + 3.81584
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: Null, Axis trans: None
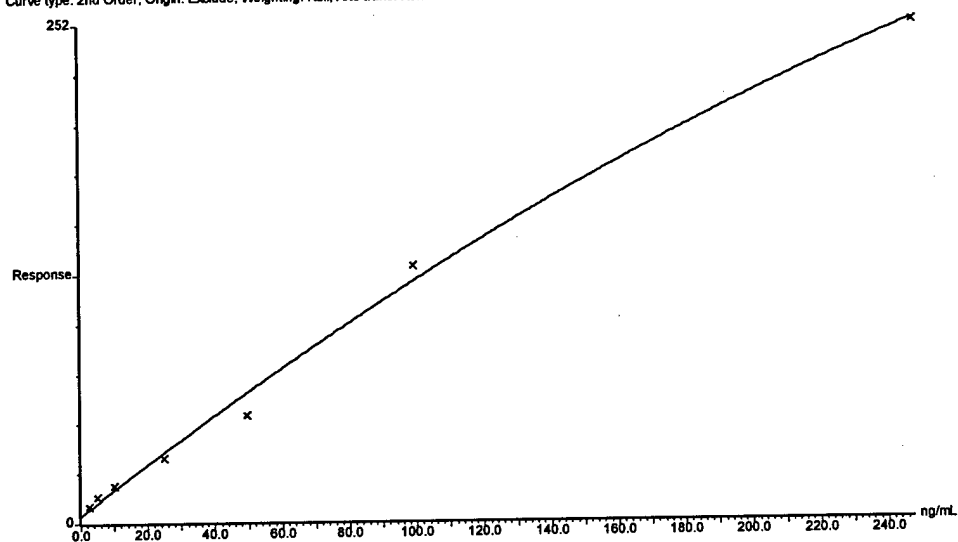
Figure 7: Regression Line for TDG in Urine Donor #3

Compound 1 name: HFBA Der. of TDG
Coefficient of Determination: 0.999929
Calibration curve: 0.000616771 * x^2 + 0.616243 * x + 8.31044
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: Null, Axis trans: None
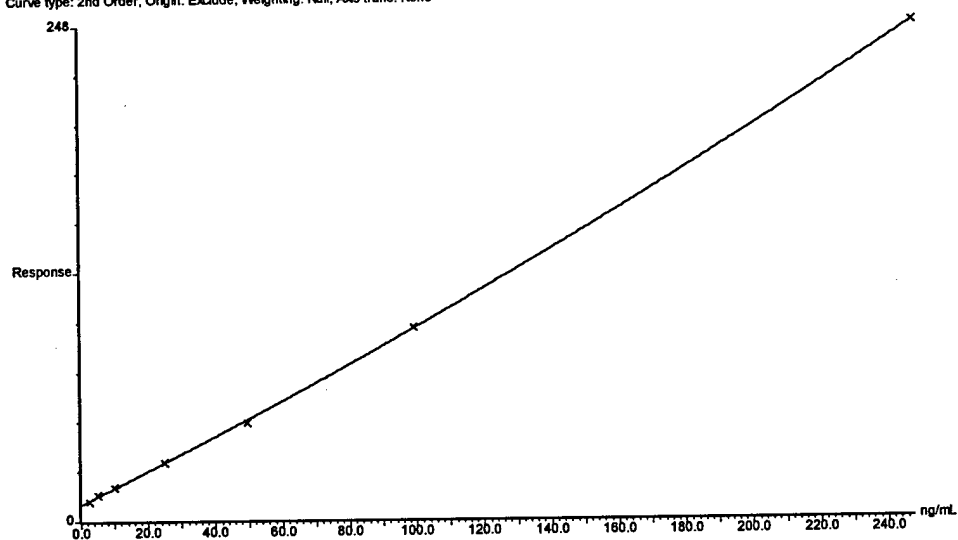
Figure 8: Regression Line for TDG in Urine Donor #4

Compound 1 name: HFBA Der. of TDG
Coefficient of Determination: 0.994477
Calibration curve: -0.000408257 * x^2 + 0.960367 * x + 15.9025
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: Null, Axis trans: None
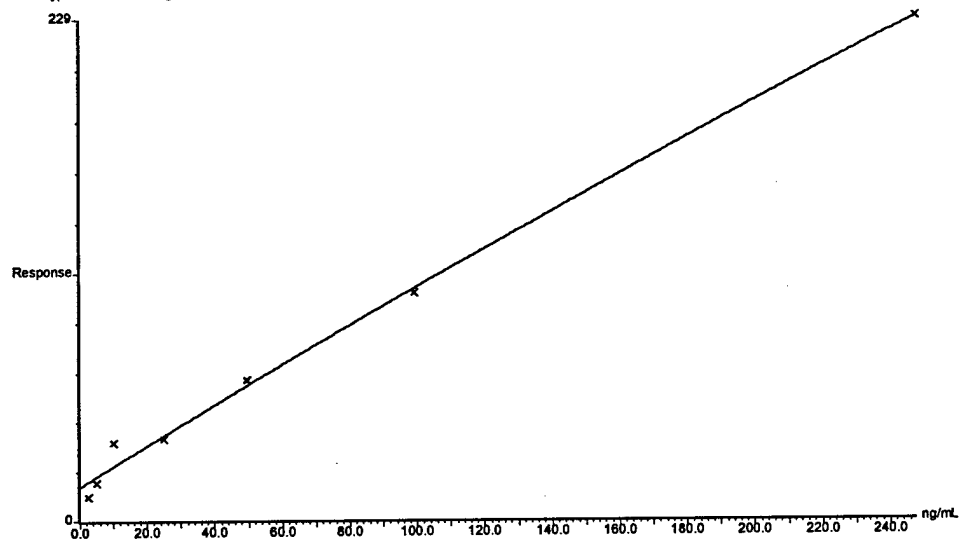
Figure 9: Regression Line for TDG in Urine Donor #5

Compound 1 name: HFBA Der. of TDG
Coefficient of Determination: 0.999633
Calibration curve: -5.26038e-5 * x^2 + 1.01017 * x + 5.54888
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: Null, Axis trans: None
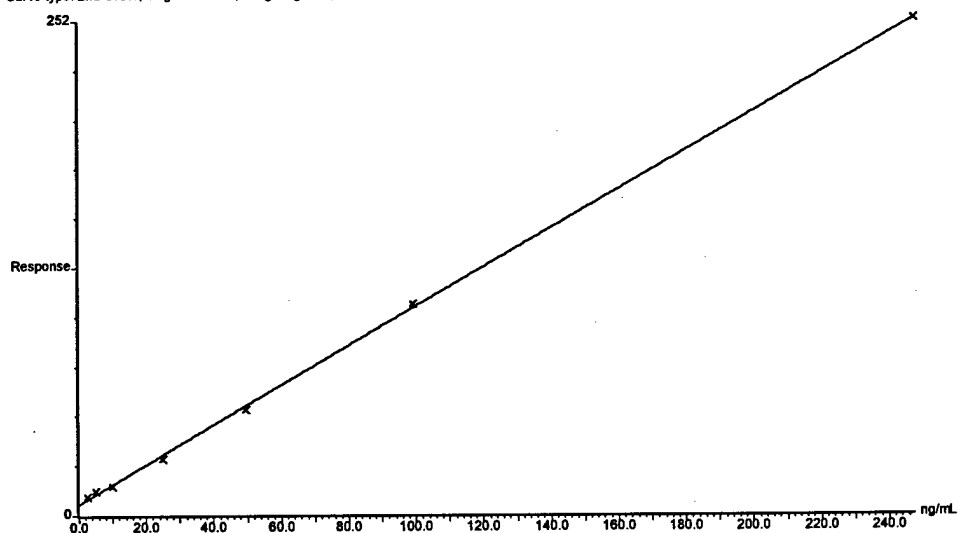
Figure 10: Regression Line for TDG in Urine Donor #6

Compound 1 name: HFBA Der. of TDG
Coefficient of Determination: 0.999521
Calibration curve: -0.000883154 * x^2 + 1.25639 * x + 5.73216
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: Null, Axis trans: None
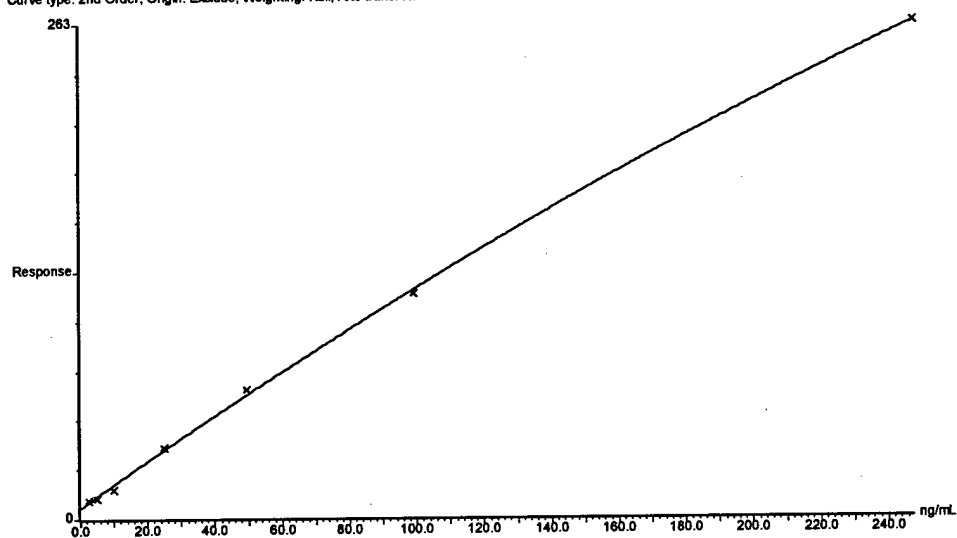
Figure 11: Regression Line for TDG in Urine Donor #7

Table 3. Calculated Concentrations of TDG

Thiodiglycol (ng/ml)	Calculated Concentrations (ng/ml)					Average	Percent Difference Between Calculated and Spiked	STDEV	% RSD
	Trial 1 3/12/99	Trial 2 3/16/99	Trial 3 3/22/99	Trial 4 3/23/99	Trial 5 3/25/99				
WATER									
247.5	247.2	248.0	247.6	247.4	247.6	247.5	0.0%	0.3	0.1%
99.0	101.6	95.8	99.0	100.9	99.5	99.4	0.4%	2.2	2.3%
49.5	49.3	52.7	47.2	44.3	43.3	47.4	4.3%	3.8	8.0%
24.8	19.9	28.1	29.5	28.6	32.8	27.8	12.0%	4.8	17.2%
9.90	4.8	8.2	8.2	11.1	14.2	9.3	6.2%	3.5	37.9%
4.95	0.5	2.7	5.6	0.0	8.7	3.5	29.0%	3.6	103.3%
2.48	14.9	2.6	1.0	0.9	0.0	3.9	56.9%	6.2	159.7%
URINE DONOR 1									
247.5	247.3	247.3	247.1	247.6	247.1	247.3	0.1%	0.2	0.1%
99.0	101.4	100.7	102.8	98.1	101.9	101.0	2.0%	1.8	1.8%
49.5	45.4	47.2	45.1	51.1	46.2	47.0	5.0%	2.4	5.2%
24.8	25.5	24.4	23.0	23.3	23.3	23.9	3.7%	1.0	4.4%
9.90	10.1	9.0	8.7	12.9	10.1	10.2	2.7%	1.6	16.0%
4.95	5.0	5.3	4.9	4.0	5.8	5.0	1.4%	0.7	13.0%
2.48	3.4	4.2	6.6	1.2	3.7	3.8	53.3%	1.9	51.0%
URINE DONOR 2									
247.5	247.5	247.4	248.7	247.9	247.0	247.7	0.1%	0.7	0.3%
99.0	97.7	100.5	91.0	97.4	106.2	98.5	0.5%	5.5	5.6%
49.5	56.5	46.8	55.6	51.9	39.1	50.0	0.9%	7.2	14.4%
24.8	15.7	25.6	34.5	24.5	19.7	24.0	3.3%	7.1	29.4%
9.90	9.8	10.1	10.3	7.4	4.8	8.5	14.4%	2.3	27.6%
4.95	4.3	7.6	5.1	9.7	16.1	8.6	72.8%	4.7	55.3%
2.48	6.7	0.3	0.0	0.0	4.8	2.4	4.6%	3.2	133.3%
URINE DONOR 3									
247.5	247.1	246.4	247.0	247.5	247.3	247.1	0.2%	0.4	0.2%
99.0	102.7	106.4	103.3	99.1	101.6	102.6	3.6%	2.7	2.6%
49.5	44.1	40.1	44.6	49.1	45.8	44.7	9.6%	3.2	7.2%
24.8	23.8	22.8	21.7	24.7	23.0	23.2	6.5%	1.1	4.8%
9.90	13.3	11.6	11.7	12.3	12.1	12.2	23.1%	0.7	5.4%
4.95	0.8	7.5	2.1	3.8	8.3	4.5	9.3%	3.3	72.7%
2.48	6.3	3.5	7.8	1.8	0.1	3.9	57.3%	3.2	81.6%
URINE DONOR 4									
247.5	247.3	247.4	247.2	247.4	247.3	247.3	0.1%	0.1	0.0%
99.0	101.2	99.8	101.8	100.1	100.7	100.7	1.7%	0.8	0.8%
49.5	46.5	48.0	45.2	47.8	47.9	47.1	4.9%	1.2	2.6%
24.8	24.5	25.0	24.9	25.4	21.9	24.3	1.9%	1.4	5.7%
9.90	9.7	10.3	9.2	8.2	12.8	10.0	1.5%	1.7	17.3%
4.95	4.5	5.8	8.1	5.4	4.7	5.7	14.6%	1.5	25.7%
2.48	4.6	1.8	1.8	3.9	2.8	3.0	19.5%	1.2	41.9%
URINE DONOR 5									
247.5	247.3	247.9	247.5	247.4	247.2	247.5	0.0%	0.3	0.1%
99.0	100.8	96.4	99.6	99.7	101.8	99.7	0.7%	2.0	2.0%
49.5	48.7	52.1	45.0	49.0	44.2	47.8	3.4%	3.2	6.7%
24.8	22.1	23.34	33.0	23.5	26.9	25.8	3.9%	4.4	17.1%
9.90	6.9	21.5	5.8	9.9	10.3	10.9	10.0%	6.3	57.4%
4.95	7.5	1.7	4.2	6.5	3.9	4.7	4.2%	2.3	48.0%
2.48	5.0	0.0	3.1	2.1	3.8	2.8	12.7%	1.9	67.2%
URINE DONOR 6									
247.5	247.1	247.3	247.4	247.4	247.4	247.3	0.1%	0.2	0.1%
99.0	102.2	101.0	100.2	99.9	99.9	100.6	1.6%	1.0	1.0%
49.5	46.8	47.7	47.8	47.7	48.3	47.6	3.8%	0.5	1.1%
24.8	21.2	22.8	23.8	25.7	23.4	23.4	5.7%	1.6	7.0%
9.90	11.4	9.0	13.0	9.7	12.3	11.1	11.9%	1.7	15.1%
4.95	3.4	6.7	4.2	5.2	6.3	5.1	4.0%	1.4	27.1%
2.48	6.2	3.8	1.9	2.6	0.5	3.0	19.9%	2.1	72.0%
URINE DONOR 7									
247.5	247.1	247.8	247.2	247.3	247.3	247.3	0.1%	0.2	0.1%
99.0	102.7	97.1	101.5	101.1	101.5	100.8	1.8%	2.1	2.1%
49.5	44.7	52.0	46.0	46.2	46.2	47.0	5.0%	2.8	6.0%
24.8	22.2	26.1	24.3	24.9	23.0	24.1	2.8%	1.5	6.4%
9.90	13.5	7.7	9.6	10.2	10.2	10.2	3.5%	2.1	20.5%
4.95	3.0	4.2	7.0	5.7	7.7	5.5	11.4%	1.9	35.3%
2.48	5.0	3.3	2.6	2.7	2.2	3.2	27.2%	1.1	34.5%

Table 4.

5-DAY AVERAGES - URINE OF SEVEN DONORS

Spiked Concentration Levels of Thiodiglycol (ng/ml)	Average Thiodiglycol (ng/ml)	Percent Difference Between Calculated and Spiked	STDEV	% RSD
247.5	247.3	0.1%	0.3	0.1%
99.0	100.6	1.7%	2.3	2.3%
49.5	47.3	4.7%	3.0	6.2%
24.8	24.1	4.0%	2.6	10.7%
9.90	10.4	9.6%	2.3	22.8%
4.95	5.6	16.8%	2.2	39.6%
2.48	3.1	27.8%	2.1	68.8%

% RSD At Various Concentrations of TDG in Urine
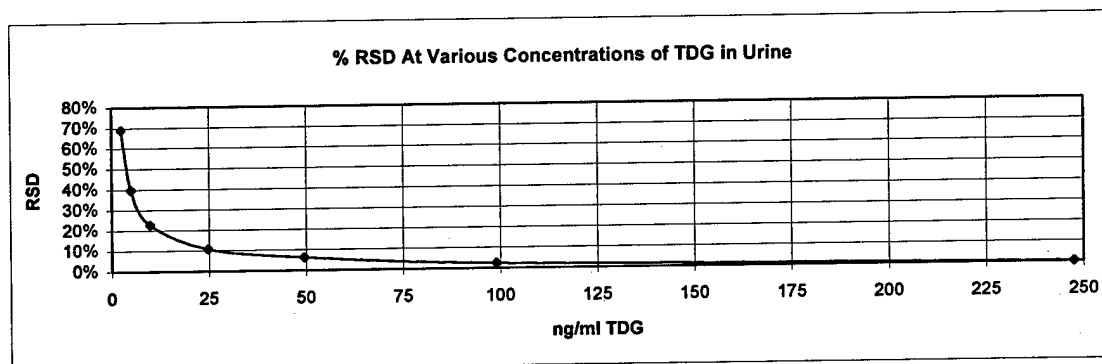
Table 5.

RELATIVE PERCENT DIFFERENCES BETWEEN SPIKED AND CALCULATED THIODIGLYCOL
CONCENTRATIONS IN URINE - CALCULATIONS PERFORMED USING WATER
STANDARDS,URINE DONOR #2 STANDARDS AND URINE DONOR #4 STANDARDS

Thiodiglycol Concentration (ng/ml)	Urine Water Stds Run 1	Urine Water Stds Run 2	Urine Water Stds Run 3	Urine Water Stds Run 4	Urine Water Stds Run 5	Average Water Stds
247.5	4.3%	14.8%	2.5%	40.3%	14.8%	15.3%
99.0	16.0%	11.5%	3.9%	46.3%	12.8%	18.1%
49.5	15.7%	10.8%	15.9%	45.3%	2.4%	18.0%
24.8	13.9%	22.8%	31.1%	41.2%	14.0%	24.6%
9.9	71.5%	56.5%	27.5%	47.4%	32.4%	47.1%
5.0	131%	87.3%	66.1%	95.7%	168%	110%
2.5	223%	141%	134%	46.1%	195%	148%

Thiodiglycol Concentration (ng/ml)	Urine Urine Donor #2 Stds Run 1	Urine Urine Donor #2 Stds Run 2	Urine Urine Donor #2 Stds Run 3	Urine Urine Donor #2 Stds Run 4	Urine Urine Donor #2 Stds Run 5	Average Urine Donor #2 Stds
247.5	3.7%	5.7%	6.6%	38.5%	8.6%	12.6%
99.0	14.4%	16.3%	20.5%	34.6%	7.6%	18.7%
49.5	48.3%	34.7%	35.8%	24.2%	47.9%	38.2%
24.8	91.7%	60.5%	44.6%	27.3%	89.0%	62.6%
9.9	87.2%	75.1%	77.0%	55.3%	94.4%	77.8%
5.0	87.1%	89.4%	81.2%	95.9%	125%	95.7%
2.5	116%	88.7%	76.0%	85.8%	94.0%	92.2%

Thiodiglycol Concentration (ng/ml)	Urine Urine Donor #4 Stds Run 1	Urine Urine Donor #4 Stds Run2	Urine Urine Donor #4 Stds Run 3	Urine Urine Donor #4 Stds Run 4	Urine Urine Donor #4 Stds Run 5	Average Urine Donor #4 Stds
247.5	2.1%	2.9%	2.4%	36.8%	4.2%	9.7%
99.0	8.8%	19.3%	6.7%	33.7%	4.0%	14.5%
49.5	14.9%	18.6%	10.4%	33.1%	6.5%	16.7%
24.8	13.2%	44.7%	29.2%	36.8%	11.4%	27.1%
9.9	69.8%	81.3%	37.3%	51.1%	47.2%	57.3%
5.0	104%	108%	71.6%	91.9%	170%	109%
2.5	269%	89.6%	185%	79.1%	213%	167%

Table 6. TDG Concentrations Using Different Quantitation Ions

Thiodiglycol (ng/ml)	Calculated Concentrations Trial 1 (241/245 m/e) 3/12/99	Percent Difference Between Calculated and Spiked	Calculated Concentrations Trial 1 (300/307 m/e) 3/12/99	Percent Difference Between Calculated and Spiked
WATER				
247.5	247.2	0.1%	247.1	0.2%
99.0	101.6	2.6%	102.6	3.6%
49.5	49.3	0.5%	47.6	3.9%
24.8	19.9	19.8%	19.9	19.7%
9.90	4.8	51.9%	5.1	48.5%
4.95	0.5	89.1%	1.7	66.7%
2.48	14.9	500%	14.2	471.8%
URINE DONOR 1				
247.5	247.3	0.1%	247.2	0.1%
99.0	101.4	2.5%	101.4	2.5%
49.5	45.4	8.2%	45.9	7.3%
24.8	25.5	2.9%	24.7	0.4%
9.90	10.1	2.2%	10.1	1.5%
4.95	5.0	1.6%	5.5	11.3%
2.48	3.4	35.1%	3.4	35.1%
URINE DONOR 2				
247.5	247.5	0.0%	247.3	0.1%
99.0	97.7	1.3%	100.8	1.9%
49.5	56.5	14.2%	49.2	0.7%
24.8	15.7	36.7%	18.2	26.6%
9.90	9.8	0.9%	16.5	66.9%
4.95	4.3	12.9%	0.0	100.0%
2.48	6.7	168.5%	6.5	160.1%
URINE DONOR 3				
247.5	247.1	0.2%	247.1	0.2%
99.0	102.7	3.7%	102.5	3.5%
49.5	44.1	10.9%	45.5	8.1%
24.8	23.8	3.9%	22.7	8.5%
9.90	13.3	33.8%	10.5	6.1%
4.95	0.8	83.0%	3.5	28.7%
2.48	6.3	155.6%	6.4	157.3%
URINE DONOR 4				
247.5	247.26	0.1%	247.3	0.1%
99.0	101.19	2.2%	101.2	2.2%
49.5	46.45	6.2%	45.8	7.5%
24.8	24.48	1.3%	25.3	1.9%
9.90	9.71	1.9%	10.8	9.0%
4.95	4.46	9.9%	4.9	0.2%
2.48	4.57	84.3%	2.9	15.7%
URINE DONOR 5				
247.5	247.26	0.1%	247.3	0.1%
99.0	100.78	1.8%	100.8	1.8%
49.5	48.73	1.6%	47.7	3.7%
24.8	22.05	11.1%	23.4	5.7%
9.90	6.89	30.4%	8.3	15.8%
4.95	7.46	50.7%	6.6	33.5%
2.48	4.97	100.4%	4.0	62.1%
URINE DONOR 6				
247.5	247.06	0.2%	247.1	0.1%
99.0	102.15	3.2%	102.3	3.4%
49.5	46.77	5.5%	45.2	8.6%
24.8	21.21	14.5%	23.3	6.0%
9.90	11.39	15.1%	10.1	1.5%
4.95	3.4	31.3%	5.9	19.2%
2.48	6.16	148.4%	4.2	69.0%
URINE DONOR 7				
247.5	247.12	0.2%	247.1	0.1%
99.0	102.7	3.7%	102.4	3.4%
49.5	44.69	9.7%	45.2	8.8%
24.8	22.18	10.6%	23.1	6.9%
9.90	13.52	36.6%	10.4	5.2%
4.95	2.96	40.2%	5.4	8.9%
2.48	4.97	100.4%	4.6	83.9%

Table 7.

SAMPLE SET CALIBRATED USING AQUEOUS STANDARDS

Thiodiglycol (ng/ml)	Calculated Conc. Water	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #1	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #2	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #3	Difference (Calculated - Actual)	% Diff.
241/245 m/e Run 1 - 3/12/99												
247.5	247.2	-0.33	0.1%	264.2	16.7	6.7%	265.9	18.4	7.4%	252.5	5.0	2.0%
99.0	101.6	2.60	2.6%	112.8	13.8	13.9%	128.7	29.7	30.0%	109.9	10.9	11.0%
49.5	49.3	-0.23	0.5%	48.6	-0.9	1.8%	87.3	37.8	76.3%	47.8	-1.7	3.4%
24.8	19.9	-4.92	19.8%	24.9	0.1	0.4%	37.6	12.8	51.6%	25.8	1.0	4.0%
9.9	4.8	-5.14	51.9%	6.3	-3.6	36.8%	38.1	28.2	285%	14.1	4.2	42.7%
5.0	0.5	-4.41	89.1%	0.0	-4.9	99.2%	19.1	14.1	286%	0.0	-5.0	100%
2.5	14.9	12.40	500%	0.0	-2.5	100%	19.0	16.5	667%	6.5	4.0	161%
300/307 m/e Run 1 - 3/12/99												
247.5	247.1	-0.42	0.2%	245.2	-2.3	0.9%	242.9	-4.6	1.9%	234.8	-12.7	5.1%
99.0	102.6	3.56	3.6%	104.3	5.3	5.3%	101.7	2.7	2.7%	102.8	3.8	3.8%
49.5	47.6	-1.91	3.9%	44.8	-4.7	9.5%	52.9	3.4	6.9%	44.9	-4.6	9.3%
24.8	19.9	-4.87	19.6%	21.2	-3.6	14.4%	21.5	-3.3	13.4%	20.7	-4.1	16.6%
9.9	5.1	-4.80	48.5%	4.6	-5.3	53.4%	22.5	12.6	127%	7.5	-2.4	24.0%
5.0	1.7	-3.30	66.7%	0.0	-5.0	100%	6.8	1.9	38.0%	0.0	-5.0	100%
2.5	14.2	11.70	472%	0.0	-2.5	100%	13.2	10.7	430%	3.0	0.6	22.2%

Thiodiglycol (ng/ml)	Calculated Conc. Donor #4	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #5	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #6	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #7	Difference (Calculated - Actual)	% Diff.
241/245 m/e Run 1 - 3/12/99												
247.5	253.4	5.9	2.4%	254.3	6.8	2.7%	259.1	11.6	4.7%	257.5	10.0	4.0%
99.0	108.0	9.0	9.1%	115.3	16.3	16.4%	118.0	19.0	19.2%	111.1	12.1	12.2%
49.5	48.5	-1.0	2.0%	59.4	9.9	19.9%	52.3	2.8	5.7%	49.2	-0.3	0.5%
24.8	23.8	-1.0	4.0%	30.5	5.7	23.1%	21.3	-3.5	14.1%	24.7	-0.1	0.3%
9.9	7.0	-3.0	29.8%	13.8	3.9	39.7%	8.6	-1.3	12.9%	15.2	5.3	53.6%
5.0	0.9	-4.1	81.8%	14.0	9.1	183%	0.0	-5.0	100%	1.7	-3.3	65.7%
2.5	0.0	-2.5	100%	11.7	9.2	372%	1.8	-0.7	28.6%	5.7	3.2	130%
300/307 m/e Run 1 - 3/12/99												
247.5	240.9	-6.6	2.7%	244.2	-3.3	1.3%	247.1	-0.4	0.2%	251.3	3.8	1.5%
99.0	102.7	3.7	3.8%	100.8	1.8	1.8%	105.6	6.6	6.7%	106.5	7.5	7.6%
49.5	44.7	-4.9	9.8%	46.9	-2.6	5.3%	44.6	-4.9	9.8%	44.8	-4.8	9.6%
24.8	22.3	-2.5	10.2%	21.8	-3.0	12.1%	20.4	-4.5	17.9%	20.2	-4.6	18.4%
9.9	6.2	-3.7	37.5%	6.2	-3.7	37.7%	5.4	-4.5	45.2%	6.0	-4.0	39.9%
5.0	0.0	-5.0	100%	4.4	-0.6	11.7%	0.7	-4.2	85.3%	0.3	-4.7	94.7%
2.5	0.0	-2.5	100%	1.7	-0.8	32.7%	0.0	-2.5	100%	0.0	-2.5	100%

Table 8.

SAMPLE SET CALIBRATED USING URINE DONOR 2 STANDARDS

Thiodiglycol (ng/ml)	Calculated Conc. Water	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #1	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #2	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #3	Difference (Calculated - Actual)	% Diff.
241/245 m/e Run 1 - 3/12/99												
247.5	225.8	-21.72	8.8%	245.4	-2.1	0.9%	247.4	-0.1	0.1%	231.9	-15.7	6.3%
99.0	71.6	-27.37	27.6%	82.7	-16.3	16.5%	98.7	-0.3	0.3%	79.8	-19.2	19.4%
49.5	21.3	-28.17	56.9%	20.7	-28.8	58.1%	57.6	8.1	16.4%	20.0	-29.5	59.6%
24.8	0.0	-24.80	100%	0.0	-24.8	100%	10.4	-14.4	57.9%	0.0	-24.8	100%
9.9	0.0	-9.90	100%	0.0	-9.9	100%	10.9	1.0	10.4%	0.0	-9.9	100%
5.0	0.0	-4.95	100%	0.0	-5.0	100%	5.4	0.5	9.7%	0.0	-5.0	100%
2.5	0.0	-2.48	100%	0.0	-2.5	100%	7.8	5.3	214%	0.0	-2.5	100%
300/307 m/e Run 1 - 3/12/99												
247.5	251.5	3.99	1.6%	249.6	2.1	0.8%	247.2	-0.3	0.1%	239.0	-8.5	3.4%
99.0	102.2	3.15	3.2%	104.0	5.0	5.0%	101.2	2.2	2.2%	102.4	3.3	3.4%
49.5	43.9	-5.60	11.3%	40.9	-8.6	17.3%	49.6	0.1	0.2%	41.0	-8.5	17.2%
24.8	14.3	-10.55	42.5%	15.7	-9.2	36.9%	15.9	-8.9	35.8%	15.1	-9.7	39.2%
9.9	0.0	-9.90	100%	0.0	-9.9	100%	17.0	7.1	71.8%	0.9	-9.0	91.2%
5.0	0.0	-4.95	100%	0.0	-5.0	100%	0.1	-4.8	97.4%	0.0	-5.0	100%
2.5	8.1	5.58	225%	0.0	-2.5	100%	6.9	4.5	180%	0.0	-2.5	100%

Thiodiglycol (ng/ml)	Calculated Conc. Donor #4	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #5	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #6	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #7	Difference (Calculated - Actual)	% Diff.
241/245 m/e Run 1 - 3/12/99												
247.5	232.9	-14.6	5.9%	234.0	-13.6	5.5%	239.4	-8.1	3.3%	237.6	-9.9	4.0%
99.0	77.9	-21.1	21.3%	85.2	-13.8	14.0%	87.9	-11.1	11.2%	81.0	-18.0	18.2%
49.5	20.6	-28.9	58.3%	30.8	-18.7	37.7%	24.2	-25.3	51.2%	21.3	-28.2	57.0%
24.8	0.0	-24.8	100%	3.9	-20.9	84.3%	0.0	-24.8	100%	0.0	-24.8	100%
9.9	0.0	-9.9	100%	0.0	-9.9	100%	0.0	-9.9	100%	0.0	-9.9	100%
5.0	0.0	-5.0	100%	0.0	-5.0	100%	0.0	-5.0	100%	0.0	-5.0	100%
2.5	0.0	-2.5	100%	0.0	-2.5	100%	0.0	-2.5	100%	0.0	-2.5	100%
300/307 m/e Run 1 - 3/12/99												
247.5	245.2	-2.3	0.9%	248.6	1.1	0.4%	251.5	4.0	1.6%	255.7	8.2	3.3%
99.0	102.3	3.3	3.4%	100.3	1.3	1.3%	105.4	6.4	6.4%	106.3	7.3	7.4%
49.5	40.8	-8.7	17.7%	43.1	-6.4	12.9%	40.7	-8.8	17.7%	40.9	-8.6	17.4%
24.8	16.8	-8.0	32.4%	16.3	-8.5	34.4%	14.7	-10.1	40.7%	14.6	-10.2	41.3%
9.9	0.0	-9.9	100%	0.0	-9.9	100%	0.0	-9.9	100%	0.0	-9.9	100%
5.0	0.0	-5.0	100%	0.0	-5.0	100%	0.0	-5.0	100%	0.0	-5.0	100%
2.5	0.0	-2.5	100%	0.0	-2.5	100%	0.0	-2.5	100%	0.0	-2.5	100%

Table 9.

SAMPLE SET CALIBRATED USING URINE DONOR 4 STANDARDS

Thiodiglycol (ng/ml)	Calculated Conc. Water	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #1	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #2	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #3	Difference (Calculated - Actual)	% Diff.
241/245 m/e Run 1 - 3/12/99												
247.5	240.6	-6.86	2.8%	258.9	11.4	4.6%	260.8	13.3	5.4%	246.3	-1.2	0.5%
99.0	95.2	-3.81	3.8%	105.7	6.7	6.8%	120.9	21.9	22.1%	103.0	4.0	4.0%
49.5	47.1	-2.38	4.8%	46.5	-3.0	6.0%	81.8	32.3	65.3%	45.8	-3.7	7.4%
24.8	21.0	-3.79	15.3%	25.4	0.6	2.5%	36.7	11.9	47.9%	26.2	1.4	5.7%
9.9	7.8	-2.09	21.1%	9.1	-0.8	8.0%	37.2	27.3	275%	16.0	6.1	61.3%
5.0	4.2	-0.80	16.2%	3.7	-1.2	24.8%	19.5	14.6	295%	4.0	-1.0	19.2%
2.5	17.1	14.60	589%	1.9	-0.5	21.8%	21.0	18.5	747%	9.3	6.8	275%
300/307 m/e Run 1 - 3/12/99												
247.5	254.4	6.90	2.8%	252.2	4.7	1.9%	249.6	2.1	0.8%	240.4	-7.1	2.9%
99.0	101.0	2.04	2.1%	102.7	3.7	3.8%	100.2	1.2	1.2%	101.2	2.2	2.3%
49.5	48.5	-0.98	2.0%	45.9	-3.6	7.2%	53.5	4.0	8.1%	46.0	-3.5	7.1%
24.8	23.1	-1.66	6.7%	24.3	-0.5	1.9%	24.6	-0.3	1.0%	23.8	-1.0	3.9%
9.9	9.8	-0.10	1.0%	9.4	-0.5	5.4%	25.5	15.6	157%	12.0	2.1	20.9%
5.0	6.7	1.78	36.0%	4.7	-0.3	6.1%	11.4	6.4	129%	5.2	0.2	4.6%
2.5	18.0	15.50	625%	2.6	0.1	3.2%	17.0	14.5	586%	8.0	5.5	221%

Thiodiglycol (ng/ml)	Calculated Conc. Donor #4	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #5	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #6	Difference (Calculated - Actual)	% Diff.	Calculated Conc. Donor #7	Difference (Calculated - Actual)	% Diff.
241/245 m/e Run 1 - 3/12/99												
247.5	247.3	-0.2	0.1%	248.3	0.8	0.3%	253.4	5.9	2.4%	251.7	4.2	1.7%
99.0	101.2	2.2	2.2%	108.1	9.1	9.2%	110.7	11.7	11.8%	104.1	5.1	5.2%
49.5	46.5	-3.1	6.2%	56.2	6.7	13.6%	49.9	0.4	0.7%	47.1	-2.4	4.9%
24.8	24.5	-0.3	1.3%	30.4	5.6	22.6%	22.3	-2.5	10.2%	25.3	0.5	1.9%
9.9	9.7	-0.2	1.9%	15.7	5.8	58.7%	11.2	1.3	12.7%	16.9	7.0	70.9%
5.0	4.5	-0.5	9.9%	19.0	14.1	284%	2.1	-2.9	58.2%	6.8	1.8	36.8%
2.5	3.0	0.5	21.0%	13.9	11.4	458%	5.2	2.7	110%	8.7	6.2	250%
300/307 m/e Run 1 - 3/12/99												
247.5	247.3	-0.2	0.1%	251.1	3.6	1.5%	254.4	6.9	2.8%	259.2	11.7	4.7%
99.0	101.2	2.2	2.2%	99.3	0.3	0.3%	104.1	5.1	5.1%	104.9	5.9	6.0%
49.5	45.8	-3.7	7.5%	47.9	-1.7	3.3%	45.8	-3.7	7.5%	45.9	-3.6	7.3%
24.8	25.3	0.5	1.9%	24.8	0.0	0.2%	23.5	-1.3	5.1%	23.4	-1.4	5.6%
9.9	10.8	0.9	8.9%	10.8	0.9	8.7%	10.1	0.2	2.0%	10.6	0.7	6.7%
5.0	4.9	0.0	0.6%	9.2	4.2	84.8%	5.9	1.0	19.4%	5.5	0.6	11.9%
2.5	2.9	0.4	16.9%	6.7	4.3	172%	4.2	1.7	67.7%	4.8	2.3	92.7%

TASK 97-55

VALIDATION OF THE TEST-MATE KIT AS A METHOD OF MONITORING BLOOD CHOLINESTERASE LEVELS

1.0 INTRODUCTION

Battelle's Medical Research and Evaluation Facility (MREF) was requested by the United States Army Medical Research Institute of Chemical Defense (USAMRICD) to validate the use of the Test-Mate™ OP Kit for monitoring of blood cholinesterase levels. The work was done under Contract DAMD 17-89-C-9050.

Analysis using the original Test-Mate™ OP Kit has previously been reported in Technical Bulletin (TB MED 296), "Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman, GF, and Cyanide," Headquarters, Department of the Army, May 1996 (Appendix A). Since that time, the manufacturer of the Test-Mate™ OP Kit, EQM Research, Inc., Cincinnati, OH, has replaced the kit with a new system reported to be more accurate and the results more reproducible. This new system is actually composed of three individual components. The AChE Erythrocyte Cholinesterase Assay Kit (Model 460) and the PChE Plasma Cholinesterase Assay Kit (Model 470) contain the necessary reagents and supplies for analysis of erythrocyte and plasma cholinesterase, respectively. The analyzer for both enzymes is called the Test-mate ChE Cholinesterase Test System (Model 400). The Model 400 kit contains not only the analyzer, but a supply of reagents to be used in the determination of AChE. For this task, Battelle used the Models 400, 460, and 470, as supplied by the manufacturer.

Insecticides, such as organophosphates and carbamates, and certain chemical warfare agents are known to cause a depletion in erythrocyte cholinesterase (AChE) and plasma cholinesterase (PChE). The amount of enzyme inhibition is related to the degree of exposure. Other methods of analysis, such as centrifugal analyzers, involve use of a relatively expensive, non-portable instrument, as well as the preparation of samples and reagent solutions that have limited shelf life. The analysis time for the centrifugal analyzer is on the order of hours. In contrast, the Test-mate analyzer is portable. The analyzer, a hand held spectrometer, weighs

10 oz and is powered by a 9V battery, or it may be connected to a standard 110V electrical line using an adapter. The kits contain all the reagents necessary for analysis. Expiration dates for reagents are between six months and one year, and a single analysis can be completed in three to four minutes. Operator training is minimal.

2.0 EXPERIMENTAL PROCEDURES

2.1 Test-mate Chemistry

The chemistry used by the Test-mate kits is based on the Ellman method[1], where acetylthiocholine (AcTC) and butyrylthiocholine (BuTC) are hydrolyzed by AChE and PChE, respectively, to yield a carboxylic acid and thiocholine. The thiocholine reacts with dithionitrobenzoic acid (DTNB) to produce a yellow colored solution. The rate of formation of the yellow color is monitored at 450 nm.

$$\textit{Cholinesterase}$$

$$\text{AcTC/BuTC} \Rightarrow \text{Acid} + \text{Thiocholine}$$

$$\text{Thiocholine} + \text{DTNB} \Rightarrow \text{TNB-thiocholine} + \text{TNB (yellow)}$$
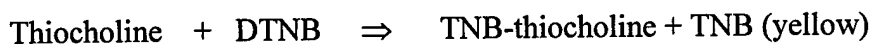
Since the method is extremely sensitive, care must be taken to avoid contamination of the samples and reagents. Erratic results were obtained while using one lot of assay buffer tubes, which was found to have been contaminated at the time of packaging by EQM. The vials had erroneously been shipped with paper cap liners instead of Teflon™ liners. In addition, it is recommended that a control blood sample be analyzed when starting an analysis with a new lot of reagent to verify that the response range is consistent with previous data.

2.2 Samples

The test kit is designed to use whole blood obtained from a finger-stick or venipuncture, however, for practical reasons the blood used in our testing was obtained only by venipuncture of seven donors. The sampling process resulted in the collection of six 7-mL vials (containing EDTA) from each donor. The blood was refrigerated (0 - 10 C) when not in use and discarded after one month.

2.3 Test-mate Equipment

Each kit, as received, contained two boxes with 48 assay buffer tubes each, one 96-well reagent plate, 100 capillary tubes (10 μL volume), 100 filter papers (Whatman 1), a plastic dropper bottle filled with 18 mL of distilled water, and two transfer pipets. The tubes, reagent plate and dropper bottle were clearly labeled with lot numbers. In addition, the transfer pipets and reagent well plate were color coded for each analysis – blue for the PChE analysis and red for the AChE analysis. Each assay buffer tube – actually a screw-cap vial – contained 2 mL of a proprietary liquid stated to be a mixture of phosphate buffer, surfactant, dye and EDTA preservative.

The reagent well plates contained different lyophilized reagents depending upon the analysis to be done, AChE or PChE. Both plates contained DTNB, phosphate buffer (pH 7.6 in analyzed solution), and Triton X-100 emulsifier. The PChE reagent well plate contained BuTC while the AChE plate had AcTC. To the AChE plate, 10-(α-diethylaminopropionyl)-phenothiazine (As1397) has also been added. The function of the As1397 is to inhibit PChE, thus increasing the specificity of the AChE analysis. Additional information concerning the reagents may be found in the package insert that comes with all kits (Appendix D).

The analyzer contains an opening into which the screw-capped vials are placed while being read. There is also an LED display which gives operator instructions, error messages, mode change and the final readings.

4

2.4 Test-mate Procedure

Detailed instructions concerning the procedure may be found in the Instruction Manual supplied with the Test-mate System[2] and in Battelle Method No. 17/Chemistry (Appendix E). A brief summary of the method, as followed by Battelle, is given here.

The Test-mate analyzer is initially zeroed with an assay buffer vial. The 10-μL capillary tube is then filled (capillary action) with the blood to be tested, ensuring visually that there are no air bubbles in the capillary. Blood on the outside of the capillary is removed by gently rolling the capillary with the index finger on a piece of filter paper supplied in the kit. The capillary tube is placed into the assay buffer vial and the vial is shaken to force the blood out of the capillary into the buffer solution. The blood from the capillary tube is readily transferred to the solution by this process. The vial is again placed in the analyzer. Three drops of distilled water is added to one reagent well and after the reagent has dissolved, this solution is transferred to the vial. The vial is again shaken and placed back in the analyzer for the final readings.

The only differences in the methods for measurement of AChE and PChE activity are the use of different reagent well plates and a different mode setting on the analyzer. As displayed on the analyzer, Hgb refers to the hemoglobin content in g/dL. The hemoglobin is measured by the analyzer during the first reading after the 10 μL of blood has been added to the assay buffer tube and before the addition of reagents from the reagent plate. Q, a hemoglobin corrected value of erythrocyte cholinesterase, is calculated by dividing the AChE result by the Hgb result.

3.0 VALIDATION

To determine the reproducibility of the method over time with multiple operators, blood samples from all seven donors were analyzed in duplicate by each of two operators on five non-consecutive days. This gives a total of twenty analyses per blood sample. Each sample was analyzed additionally by the Ellman method using a Roche Cobas Fara® centrifugal clinical chemistry analyzer.

It is of primary importance to show that the Test-mate test kit can differentiate between inhibited and non-inhibited blood, and that it can do so quantitatively. To demonstrate this, five blood samples were randomly chosen from the seven donor samples. To each of the five blood tubes was added 1 μL of neat Soman (GD). GD was chosen to inhibit the red blood cells due to its rapid aging (dealkylation) and non-reversible binding of red blood cell AChE. The tubes were inverted slowly to distribute the GD and then centrifuged to concentrate the red blood cells. The plasma was removed from each sample and discarded. The remaining red blood cells were washed five times with isotonic saline to remove excess GD. These cells were considered to be fully inhibited red blood cells.

Into thirty microcentrifuge tubes were placed six, 1-mL aliquots of non-inhibited blood from each of the five donors. These tubes were then centrifuged to pack the red blood cells. Using a positive displacement pipettor, 0, 10, 20, 30, 40, and 50 μL of non-inhibited red blood cells were removed from each of the 6 microcentrifuge tubes per donor and replaced with equal amounts of fully inhibited red blood cells previously prepared. The procedure was repeated for all five blood donor samples and the total of 30 samples were analyzed using the Test-mate System. The 30 samples also were analyzed using the Roche Cobas Fara® for comparison.

4.0 RESULTS AND DISCUSSION

The manufacturer states in their Operation Manual [2] that the accepted ranges for AChE, Q and PChE were determined using the blood of forty male and female blood bank donors between the ages of twenty and sixty, located in the midwestern United States (see Table 1). Our donors fit the same demographic description and as can be seen from the data, there is a general agreement between our values and those reported by EQM. Most differences can be attributed to a difference in sample size.

Table 1. Typical Ranges of Values for AChE and PChE

	EQM Research, Inc.			Battelle		
	Mean	STD	Range	Mean	STD	Range
AChE, U/mL	3.68	0.47	2.77-5.5	4.71	0.87	3.46-6.11
Q, U/g	27.1	2.9	21.9-37.3	34.3	4.7	31.6-41.3
PchE, U/mL	2.03	0.40	1.35-3.23	2.71	0.66	1.73-3.42

The percent of normal values that are displayed on the analyzer following each analysis are based on a later study by EQM Research which involved 100 volunteers (per conversation with Patrick Eberly, EQM Research). For example, a value of 100 percent of normal AChE corresponds with approximately 4.7 U/mL according to the analyzer. This is considerably higher than EQM's average AChE value, as listed in Table 1. It does, however, correspond closely with the value found by Battelle. The percent of normal values are constants which are set at the factory and cannot be changed by the operator. Since AChE and PChE are population dependent, the "normal" readings should be used judiciously.

Table 2 lists EQM's values for between-operator variability for ten analyses of one sample by ten operators[2] (N = 100). Figures 1-14 are graphical representations of inter-operator variability as determined in Battelle's testing.

Table 2. EQM Between Operator Variability

	AChE (U/mL)	Q (U/g)	PChE (U/mL)	Hgb (g/dL)
Mean	5.63	33.8	1.72	16.8
STD	0.21	0.8	0.15	0.5
%RSD	3.7	2.4	8.5	2.7

Tables 3-9, at the end of the text, contain the AChE data obtained from the five-day analyses of seven donor samples. The corresponding PChE data is contained in Tables 10-16. With the exception of the date, time, analyst number and statistics, the values in the tables are numbers which are displayed on the Test-mate analyzer at the end of each test. The values in Table 2 are comparable to Battelle's results, as listed at the bottom of Tables 3-16.

The linearity of each method (AChE and PChE) was confirmed in Battelle's analysis of the GD-inhibited blood samples. Tables 17-26 contain the data from these analyses, while Figures 15-24 contain the regression lines. The slopes of the regression lines are negative since there is a decrease in the amount of cholinesterase activity with an increase in the amount of inhibited red blood cells. The absolute value of the slope is an indication of the sensitivity of the method, i.e., the larger the absolute value of the slope, the easier it is to detect small changes in the value of AChE or PChE. The correlation coefficients for the regression lines vary between 0.95 and 0.99 with the average being 0.98, the standard deviation 0.01, and percent standard deviation 1.2. No comparable data was listed by the manufacturer.

The results obtained using the Roche Cobas Fara® and the five-day average AChE Test-mate values are compared in Figure 25. Although the determination using the Cobas Fara® is done on packed red blood cells, a better correlation between the two methods would have been expected (correlation coefficient 0.77). There is much better agreement for partially inhibited blood samples within donor samples, as is shown in Figures 26-30. The range of correlation coefficients here is from 0.94 to 0.99, with an average of 0.98.

5.0 CONCLUSION

The Test-mate ChE Cholinesterase Test System appears to be a quick and reproducible method for measuring relative cholinesterase inhibition. The absolute values obtained cannot, however, be interchanged with those determined by other methods. As with other methods, the ChE technique has the disadvantage of needing a baseline reading in order to determine whether an exposure has, in fact, occurred. It also has the same interferences as other methods based on Ellman's technique. The Test-mate uses whole blood, and therefore reproducible results can be obtained on the hemolyzed blood. Hemolysis can present a problem when using the Roche Cobas Fara®, since the method relies on the packing of intact red blood cells.

The Test-mate ChE Cholinesterase Test System has the advantage, by its design, of being portable and to not require reagent preparation. Only minimal operator training is necessary;

however, care must be taken not to interchange the AChE and PChE reagent well plates and to change the mode on the analyzer when switching between AChE and PChE analyses.

A suggested improvement to the system is to provide the material that is contained in the reagent wells either in a larger quantity, such as in a plastic bottle similar to the one in which the distilled water is packaged, or in the well plates, as is currently done. This would present a time savings when numerous samples are to be analyzed. In addition, problems with the disposable pipet used to transfer the reagent to the vial – e.g., bending of the pipet tip, difficulty with removing all of the solution from the pipet bulb - would be eliminated by this modification. If only a small number of samples are to be analyzed, the well plate could be used, thus eliminating solution stability concerns.

6.0 REFERENCES

1. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961; 7-88-95.

2. Test-mate ChE cholinesterase Test System (Model 400) Instruction Manual, EQM Research, Inc., 2585 Montana Avenue, Cincinnati, Ohio 45211. Rev. F2 © 1998.

3. MREF Method No. 33/In Vitro.

4. Technical Bulletin (TB MED 296), "Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman, GF, and Cyanide," Headquarters, Department of the Army, May 1996.

Table 3. AChE Analysis of Sample 387-24

Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
73198	1057	1	4.69	100	13.7	91	34.3	109
73198	1103	1	4.57	97	13.5	90	33.8	108
73198	1109	2	4.43	94	13.9	92	32.0	102
73198	1116	2	4.70	100	13.6	91	34.7	110
8398	809	1	4.46	95	13.8	92	32.4	103
8398	814	1	4.64	99	13.8	92	33.5	107
8398	816	2	4.57	97	13.5	90	33.8	108
8398	820	2	4.70	100	13.8	92	34.1	109
8598	845	1	4.54	96	14.0	93	32.5	104
8598	849	1	4.58	97	13.8	92	33.1	105
8598	814	2	4.70	100	13.6	91	34.5	110
8598	820	2	4.73	100	14.5	96	32.7	104
8798	816	1	4.55	97	13.9	92	32.8	105
8798	821	1	4.60	98	13.8	92	33.3	106
8798	826	2	4.70	100	14.0	93	33.6	107
8798	830	2	4.72	100	14.0	93	33.7	107
81098	818	1	4.52	96	13.6	91	33.2	106
81098	824	1	4.59	97	13.8	92	33.2	106
81098	828	2	4.66	99	13.9	93	33.5	107
81098	832	2	4.53	96	13.8	92	32.9	105

AVG =   4.61   13.8   33.4

STD =   0.09   0.2   0.7

%RSD =   1.9%   1.6%   2.1%

Table 4. AChE Analysis of Sample 387-28

Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
73198	1120	1	4.30	91	12.7	85	33.8	108
73198	1125	1	4.30	91	12.8	85	33.6	107
73198	1130	2	4.29	91	12.6	84	34.1	109
73198	1136	2	4.31	92	12.6	84	34.2	109
80398	830	1	3.90	83	12.4	82	31.6	101
80398	834	1	4.29	91	12.5	83	34.4	110
80398	839	2	4.08	87	12.5	83	32.7	104
80398	844	2	4.24	90	12.5	83	34.0	108
80598	855	1	4.41	94	12.6	84	35.0	112
80598	858	1	4.32	92	12.6	84	34.2	109
80598	825	2	4.06	86	12.9	86	31.4	100
80598	829	2	3.99	85	13.5	90	29.5	94
80798	840	1	4.27	91	13.0	87	32.8	105
80798	845	1	4.31	91	12.9	86	33.3	106
80798	850	2	4.36	93	12.9	86	33.9	108
80798	854	2	4.24	90	12.8	86	33.1	105
81098	842	1	4.23	90	12.7	84	33.4	106
81098	845	1	4.21	89	12.8	86	32.8	104
81098	849	2	4.03	86	12.5	84	32.2	102
81098	854	2	4.27	91	12.9	86	33.1	106

AVG =   4.22   12.7   33.2

STD =   0.14   0.2   1.3

%RSD =   3.2%   2.0%   3.8%

Table 5. AChE Analysis of Sample 387-32

Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
73198	1141	1	6.05	128	14.8	99	40.9	130
73198	1146	1	6.17	131	14.7	98	42.0	134
73198	1150	2	6.23	132	14.9	99	41.8	133
73198	1154	2	5.91	125	14.2	94	41.8	133
80398	847	1	5.84	124	14.6	97	40.0	127
80398	852	1	5.96	127	14.7	98	40.5	129
80398	857	2	6.15	131	14.6	97	42.2	134
80398	903	2	6.05	128	14.5	97	41.6	133
80598	903	1	5.97	127	15.0	100	39.8	127
80598	907	1	6.25	133	14.9	99	42.0	134
80598	835	2	6.47	137	15.1	101	42.9	136
80598	838	2	6.11	130	14.9	99	41.1	131
80798	858	1	6.08	129	14.9	99	40.9	130
80798	906	1	6.05	128	14.9	100	40.5	129
80798	911	2	6.32	134	15.1	101	41.7	133
80798	915	2	6.22	132	14.9	100	41.7	133
81098	859	1	6.03	128	14.8	99	40.8	130
81098	904	1	6.08	129	15.0	100	40.6	129
81098	909	2	6.12	130	14.7	98	41.6	133
81098	915	2	6.12	130	15.0	100	40.7	130

	AChE	Hgb	Q
AVG =	6.11	14.8	41.3
STD =	0.14	0.2	0.8
%RSD =	2.4%	1.5%	1.9%

Table 6. AChE Analysis of Sample 387-36

Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
73198	1159	1	3.51	74	12.6	84	27.8	88
73198	1204	1	3.42	73	12.3	82	27.7	88
73198	1209	2	3.51	74	12.7	85	27.6	88
73198	1213	2	3.52	75	12.5	83	28.2	90
80398	908	1	3.53	75	12.8	85	27.7	88
80398	912	1	3.49	74	12.5	84	27.9	89
80398	917	2	3.43	73	12.2	82	28.1	89
80398	922	2	3.42	73	12.7	85	26.9	86
80598	912	1	3.60	76	12.5	84	28.7	91
80598	918	1	3.48	74	12.5	84	27.8	88
80598	924	2	3.44	73	12.3	82	28.0	89
80598	928	2	3.32	71	12.5	83	26.6	85
80798	920	1	3.40	72	12.6	84	27.0	86
80798	924	1	3.43	73	12.7	84	27.0	86
80798	928	2	3.47	74	12.5	84	27.7	88
80798	934	2	3.56	76	12.9	86	27.6	88
81098	920	1	3.41	72	12.6	84	27.1	86
81098	925	1	3.40	72	12.7	85	26.8	85
81098	930	2	3.45	73	12.7	85	27.1	86
81098	934	2	3.36	71	12.5	83	26.9	86

AVG =   3.46   12.6   27.5
STD =   0.07   0.2   0.6
%RSD =   2.0%   1.4%   2.0%

Table 7. AChE Analysis of Sample 387-40

Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
73198	1217	1	4.14	88	12.8	85	32.3	103
73198	1222	1	4.32	92	13.5	90	32.0	102
73198	1226	2	4.27	91	13.5	90	31.6	101
73198	1233	2	4.29	91	13.6	91	31.6	100
80398	927	1	4.29	91	13.6	91	31.6	100
80398	931	1	4.45	95	13.5	90	33.1	105
80398	936	2	4.26	90	13.5	90	31.7	101
80398	941	2	4.24	90	13.5	90	31.4	100
80598	935	1	4.08	87	13.4	89	30.5	97
80598	940	1	4.29	91	13.6	91	31.5	100
80598	943	2	4.28	91	13.6	91	31.5	100
80598	949	2	4.38	93	13.7	91	32.0	102
80798	938	1	4.20	89	13.7	91	30.6	98
80798	944	1	4.37	93	13.8	92	31.7	101
80798	948	2	4.34	92	13.7	91	31.7	101
80798	952	2	4.34	92	13.5	90	32.2	102
81098	940	1	4.16	88	13.7	91	30.5	97
81098	944	1	4.04	86	13.3	89	30.4	97
81098	948	2	4.25	90	13.6	90	31.4	100
81098	953	2	4.18	89	13.2	88	31.7	101

AVG = 4.26   13.5   31.6

STD = 0.10   0.2   0.7

%RSD = 2.4%   1.6%   2.1%

Table 8. AChE Analysis of Sample 387-44

Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
73198	1236	1	4.89	104	14.7	98	33.2	106
73198	1242	1	4.91	104	14.9	100	32.8	105
73198	1245	2	4.77	101	14.8	99	32.2	103
73198	1250	2	4.77	101	14.4	96	33.0	105
80398	954	1	4.79	102	14.8	98	32.4	103
80398	959	1	5.09	108	15.1	100	33.8	108
80398	945	2	4.81	102	14.5	97	33.1	105
80398	950	2	4.88	104	14.5	97	33.6	107
80598	952	1	4.88	104	14.7	98	33.2	106
80598	959	1	5.13	109	14.8	99	34.6	110
80598	1003	2	5.04	107	14.9	99	33.8	108
80598	1007	2	5.03	107	14.8	98	34.0	108
80798	956	1	4.98	106	14.5	97	34.4	109
80798	1000	1	4.86	103	14.8	99	32.8	104
80798	1007	2	4.93	105	14.5	97	34.0	108
80798	1011	2	4.99	106	14.8	99	33.7	107
81098	958	1	4.84	103	14.8	99	32.7	104
81098	1002	1	4.86	103	14.7	98	33.1	105
81098	1006	2	4.91	104	14.6	97	33.5	107
81098	1011	2	4.91	104	14.7	98	33.5	107

AVG =   4.91   14.7   33.4
STD =   0.10   0.2   0.6
%RSD =   2.1%   1.2%   1.9%

Table 9. AChE Analysis of Sample 387-48

Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
73198	1254	1	5.19	110	13.4	89	38.8	123
73198	1259	1	5.44	115	13.7	91	39.6	126
73198	1303	2	4.95	105	13.8	92	35.9	114
73198	1308	2	4.88	104	13.2	88	36.8	117
80398	1012	1	5.62	119	13.7	92	40.9	130
80398	1017	1	5.67	120	13.6	90	41.8	133
80398	1003	2	5.41	115	13.4	89	40.4	129
80398	1008	2	5.52	117	13.8	92	40.1	128
80598	1008	1	5.57	118	13.7	92	40.5	129
80598	1017	1	5.35	114	13.7	92	38.9	124
80598	1022	2	5.49	117	14.1	94	39.0	124
80598	1027	2	5.19	110	13.7	91	37.9	121
80798	1015	1	5.60	119	13.7	91	40.9	130
80798	1020	1	5.50	117	14.0	93	39.3	125
80798	1025	2	5.79	123	13.7	92	42.2	134
80798	1029	2	5.76	122	14.3	95	40.3	128
81098	1016	1	5.22	111	13.7	91	38.2	122
81098	1020	1	5.31	113	13.6	90	39.1	125
81098	1025	2	5.26	112	13.2	88	39.8	127
81098	1029	2	5.46	116	13.8	92	39.5	126

AVG=	5.42	13.7	39.5
STD=	0.24	0.3	1.5
%RSD=	4.4%	2.1%	3.8%

Table 10. PChE Analysis of Sample 387-24

Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
73198	1348	1	3.44	135	13.8	92
73198	1357	1	3.38	133	13.7	91
73198	1340	2	3.54	139	13.8	92
73198	1345	2	3.42	134	14.5	97
80398	1035	1	3.27	128	13.7	91
80398	1040	1	3.31	130	13.6	90
80398	1046	2	3.51	138	13.7	91
80398	1051	2	3.39	133	13.9	93
80598	1044	1	3.28	129	13.5	90
80598	1048	1	3.39	133	13.6	91
80598	1054	2	3.51	137	13.8	92
80598	1058	2	3.47	136	13.7	92
80798	1050	1	3.53	138	14.4	96
80798	1100	1	3.46	136	13.7	91
80798	1105	2	3.45	135	13.9	93
80798	1110	2	3.39	133	13.8	92
81098	1051	1	3.46	136	13.8	92
81098	1056	1	3.55	139	14.1	94
81098	1100	2	3.32	130	13.9	92
81098	1104	2	3.41	134	14.0	94

AVG=   3.42   13.8

STD=   0.08   0.3

%RSD=   2.5%   1.8%

Table 11. PChE Analysis of Sample 387-28

Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
73198	1406	1	1.79	70	12.7	85
73198	1415	1	1.77	69	12.8	85
73198	1356	2	1.80	71	12.7	84
73198	1401	2	1.83	72	12.7	85
8398	1055	1	1.68	66	12.4	83
8398	1059	1	1.72	67	12.4	83
8398	1104	2	1.74	68	12.4	83
8398	1110	2	1.67	66	12.4	83
8598	1102	1	1.69	66	12.8	86
8598	1107	1	1.74	68	13.0	87
8598	1114	2	1.75	69	12.5	83
8598	1118	2	1.67	66	12.7	85
8798	1114	1	1.70	67	12.9	86
8798	1118	1	1.77	70	12.9	86
8798	1123	2	1.74	68	12.8	85
8798	1127	2	1.77	70	12.6	84
81098	1109	1	1.63	64	12.6	84
81098	1113	1	1.71	67	12.6	84
81098	1117	2	1.68	66	12.9	86
81098	1121	2	1.70	67	12.8	86

AVG=   1.73   12.7
STD=   0.05   0.2
%RSD=   3.0%   1.5%

Table 12. PChE Analysis of Sample 387-32

Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
73198	1427	1	2.10	82	14.8	99
73198	1433	1	2.16	85	14.8	98
73198	1417	2	2.12	83	14.8	99
73198	1422	2	2.04	80	14.3	95
8398	1115	1	2.05	80	14.4	96
8398	1120	1	2.06	81	14.6	97
8398	1125	2	2.01	79	14.9	99
8398	1129	2	2.07	81	14.6	97
8598	1124	1	2.03	80	14.7	98
8598	1129	1	2.08	82	14.9	99
8598	1132	2	2.02	79	14.6	97
8598	1137	2	1.94	76	14.7	98
8798	1130	1	2.12	83	14.9	99
8798	1136	1	2.18	85	15.3	102
8798	1141	2	1.93	76	15.3	102
8798	1145	2	2.14	84	15.5	103
81098	1125	1	2.03	80	14.9	100
81098	1131	1	2.04	80	15.0	100
81098	1135	2	1.93	76	15.1	101
81098	1139	2	2.08	82	14.8	99

AVG=   2.06   14.8

STD=   0.07   0.3

%RSD=   3.4%   2.0%

Table 13. PChE Analysis of Sample 387-36

Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
73198	1445	1	2.74	108	12.6	84
73198	1449	1	2.78	109	12.7	85
73198	1436	2	2.88	113	12.5	83
73198	1440	2	2.78	109	12.2	81
8398	1134	1	2.92	115	12.7	84
8398	1137	1	2.76	108	12.5	83
8398	1142	2	2.86	112	12.6	84
8398	1147	2	2.88	113	12.7	85
8598	1144	1	2.79	109	12.5	83
8598	1150	1	2.75	108	12.6	84
8598	1154	2	2.67	105	12.8	85
8598	1159	2	2.78	109	12.7	85
8798	1148	1	2.92	114	13.6	91
8798	1153	1	2.80	110	12.4	83
8798	1156	2	2.62	103	12.6	84
8798	1159	2	2.94	115	13.0	87
81098	1158	1	2.85	112	12.7	85
81098	1204	1	2.81	110	12.6	84
81098	1209	2	2.67	105	12.6	84
81098	1213	2	2.80	110	12.7	84

AVG=   2.80   12.7

STD=   0.09   0.3

%RSD=   3.1%   2.1%

Table 14. PChE Analysis of Sample 387-40

Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
73198	1503	1	2.24	88	13.5	90
73198	1505	1	2.59	102	13.4	89
73198	1453	2	2.53	99	13.5	90
73198	1500	2	2.54	99	13.2	88
8398	1152	1	2.61	102	13.6	91
8398	1156	1	2.48	97	13.2	88
8398	1201	2	2.53	99	13.5	90
8398	1206	2	2.01	79	13.7	91
8598	1204	1	2.39	94	13.5	90
8598	1206	1	2.36	92	13.0	87
8598	1215	2	2.26	89	12.8	86
8598	1221	2	2.49	98	13.4	89
8798	1203	1	2.49	98	13.5	90
8798	1206	1	2.54	99	13.6	90
8798	1210	2	2.42	95	13.6	90
8798	1214	2	2.46	96	13.7	91
81098	1218	1	2.42	95	13.2	88
81098	1223	1	2.35	92	13.3	88
81098	1227	2	2.38	93	13.3	89
81098	1232	2	2.44	96	13.1	88

AVG=   2.43   13.4

STD=   0.14   0.2

%RSD=   5.7%   1.8%

Table 15. PChE Analysis of Sample 387-44

Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
73198	1520	1	3.52	138	14.9	99
73198	1525	1	3.41	134	14.6	98
73198	1511	2	3.54	139	15.1	100
73198	1516	2	3.55	139	14.7	98
8398	1211	1	3.48	136	14.3	96
8398	1215	1	3.31	130	14.5	96
8398	1220	2	3.29	129	14.6	97
8398	1224	2	3.37	132	14.5	97
8598	1231	1	3.61	142	15.0	100
8598	1235	1	3.32	130	14.5	96
8598	1240	2	3.30	129	14.7	98
8598	1244	2	3.28	128	14.6	98
8798	1212	1	3.29	129	14.6	97
8798	1220	1	3.62	142	15.1	101
8798	1224	2	3.28	129	14.7	98
8798	1228	2	3.37	132	15.1	101
81098	1237	1	3.50	137	14.7	98
81098	1241	1	3.50	137	15.0	100
81098	1246	2	3.28	129	14.5	97
81098	1250	2	3.42	134	14.6	97

AVG=			3.41		14.7
STD=			0.12		0.2
%RSD=			3.5%		1.6%

Table 16. PChE Analysis of Sample 387-48

Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
73198	1537	1	3.14	123	13.7	91
73198	1543	1	3.53	139	13.7	91
73198	1528	2	3.07	120	13.7	91
73198	1533	2	3.01	118	13.7	91
8398	1227	1	3.17	124	13.3	89
8398	1232	1	3.07	120	13.7	91
8398	1237	2	3.13	123	13.8	92
8398	1243	2	3.22	126	14.0	93
8598	1255	1	3.03	119	13.8	92
8598	1259	1	3.02	118	13.9	93
8598	1304	2	3.04	119	13.6	91
8598	1309	2	3.12	122	13.5	90
8798	1232	1	3.39	133	14.8	99
8798	1237	1	3.19	125	14.0	94
8798	1239	2	3.11	122	14.2	95
8798	1244	2	3.23	127	13.9	93
81098	1254	1	3.17	124	13.7	92
81098	1259	1	3.20	125	13.9	93
81098	1303	2	2.91	114	13.7	92
81098	1308	2	3.06	120	13.7	91

AVG= 3.14   13.8

STD= 0.14   0.3

%RSD= 4.4%   2.2%

FIGURE 1.

AChE (387-24)
ANALYSES PERFORMED OVER 5 DAY PERIOD
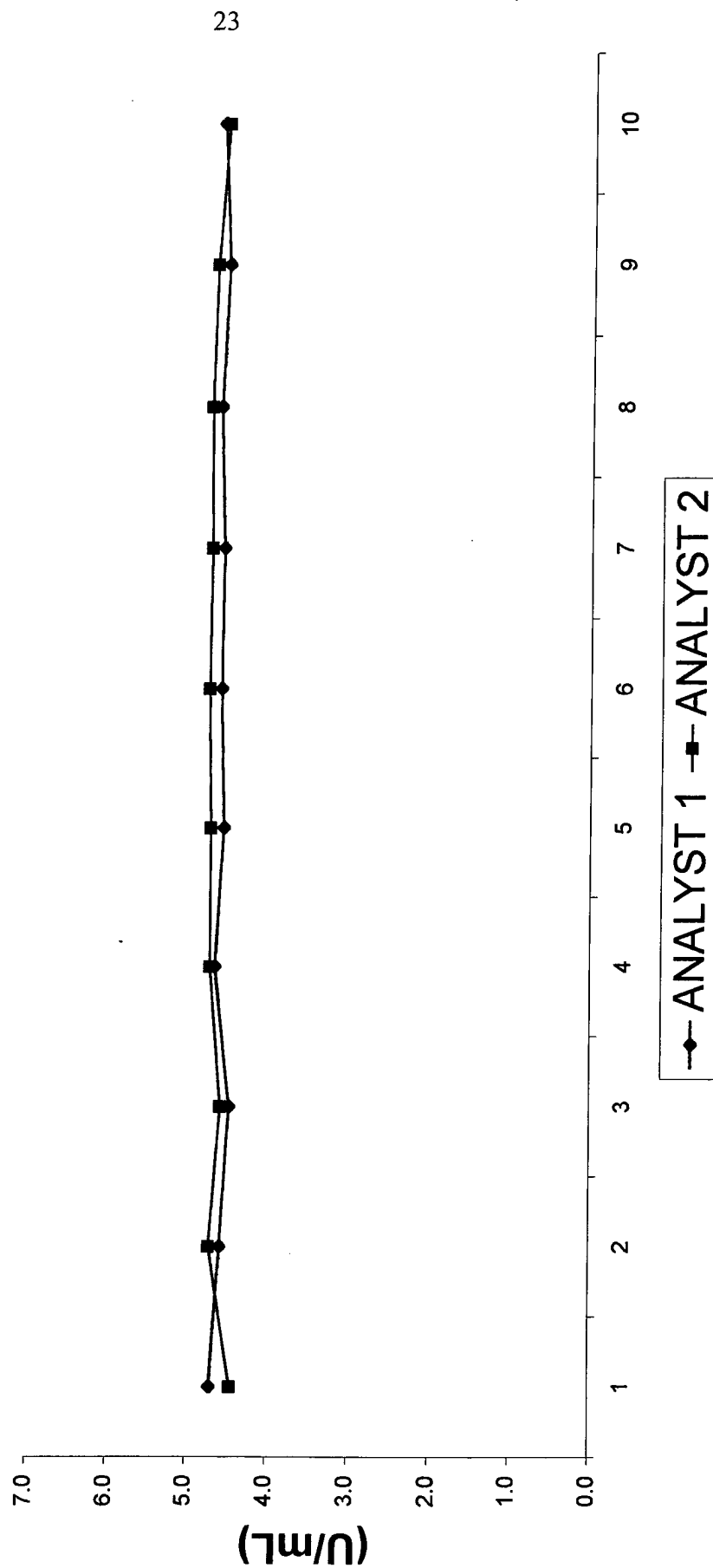
FIGURE 2.

AChE (387-28)
ANALYSES PERFORMED OVER 5 DAY PERIOD

ANALYST 1 ANALYST 2
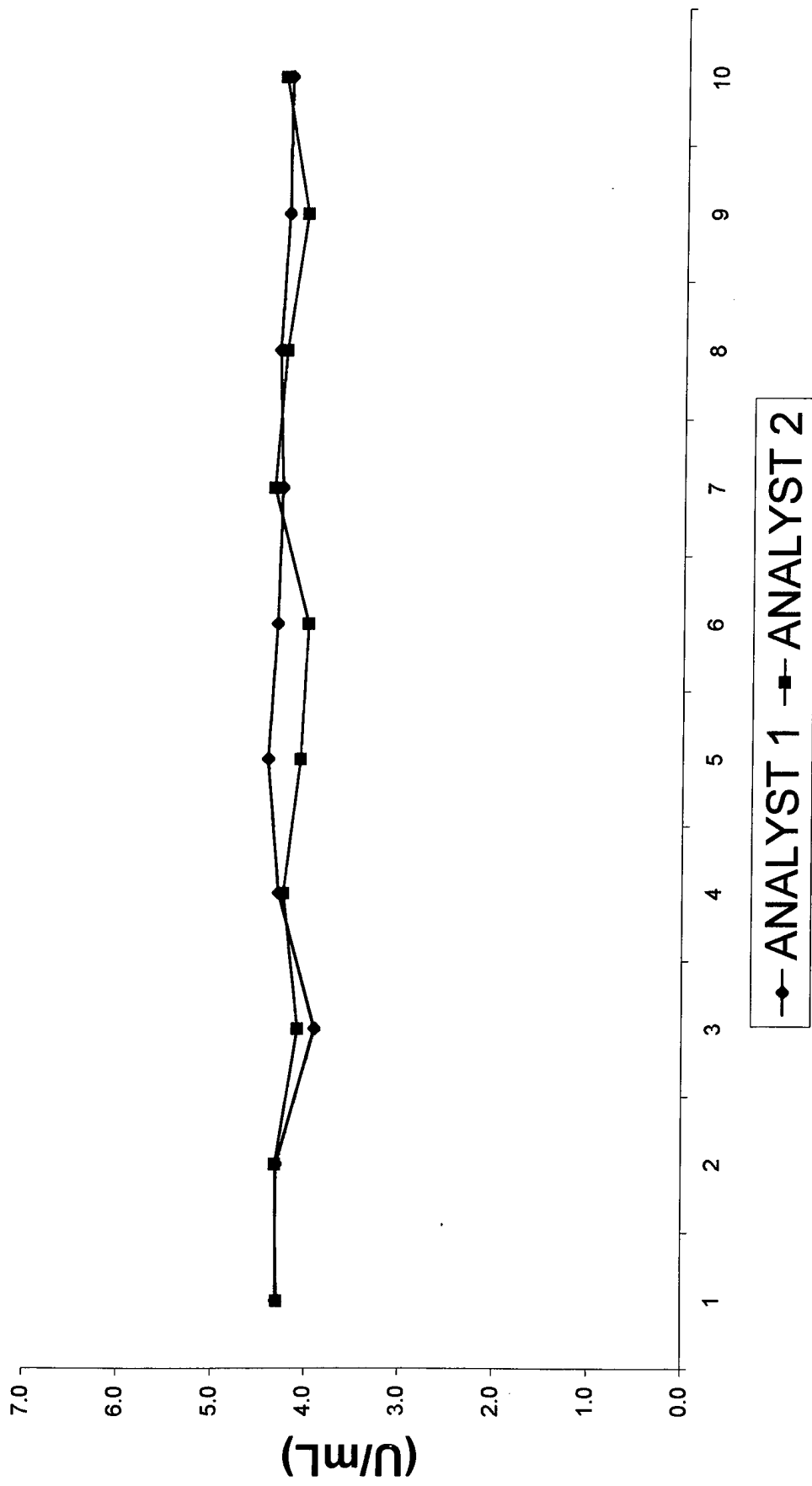
FIGURE 3.

AChE (387-32)
ANALYSES PERFORMED OVER 5 DAY PERIOD
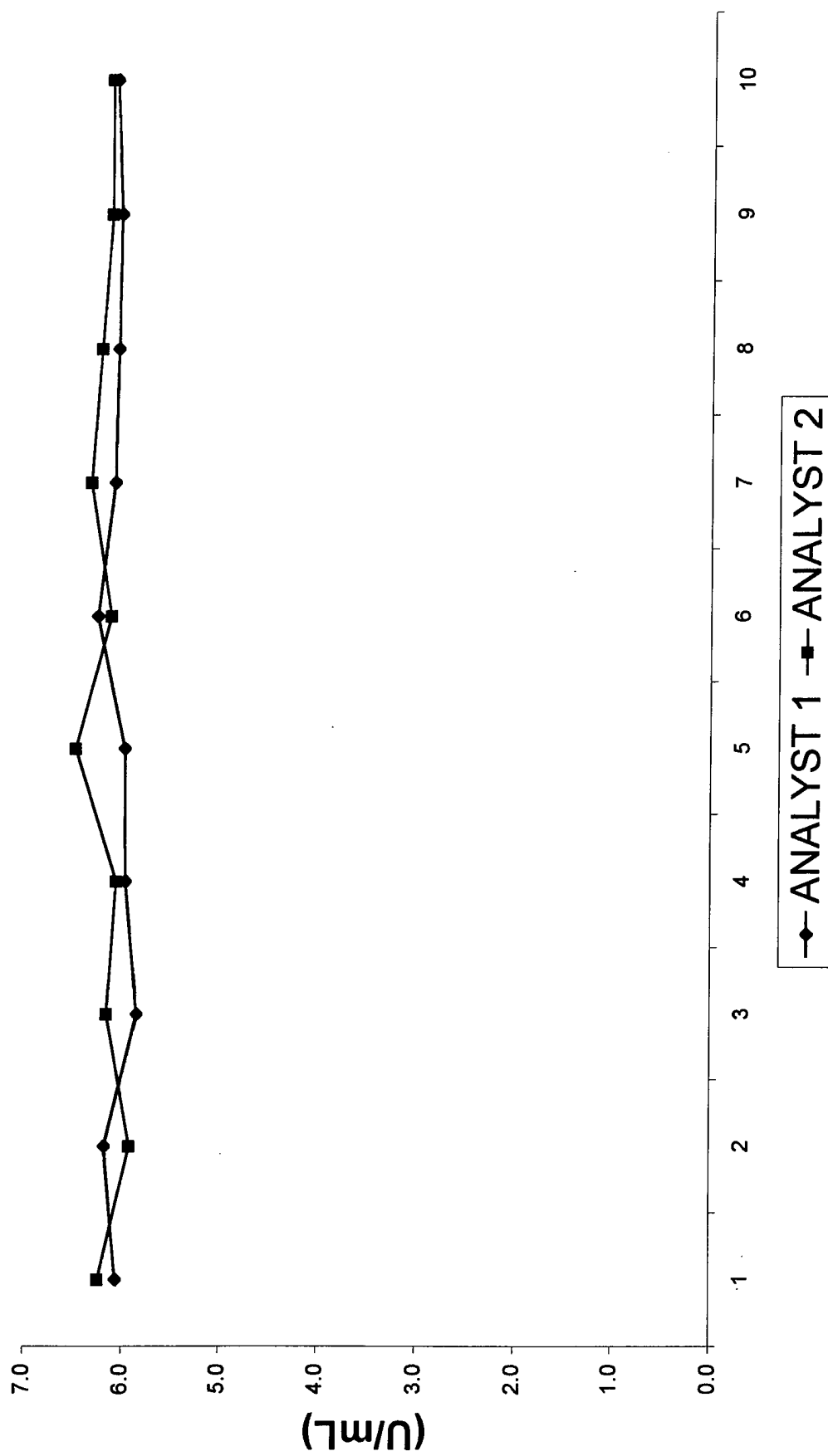
FIGURE 4.

AChE (387-36)
ANALYSES PERFORMED OVER 5 DAY PERIOD

←— ANALYST 1 ■— ANALYST 2

(U/mL)
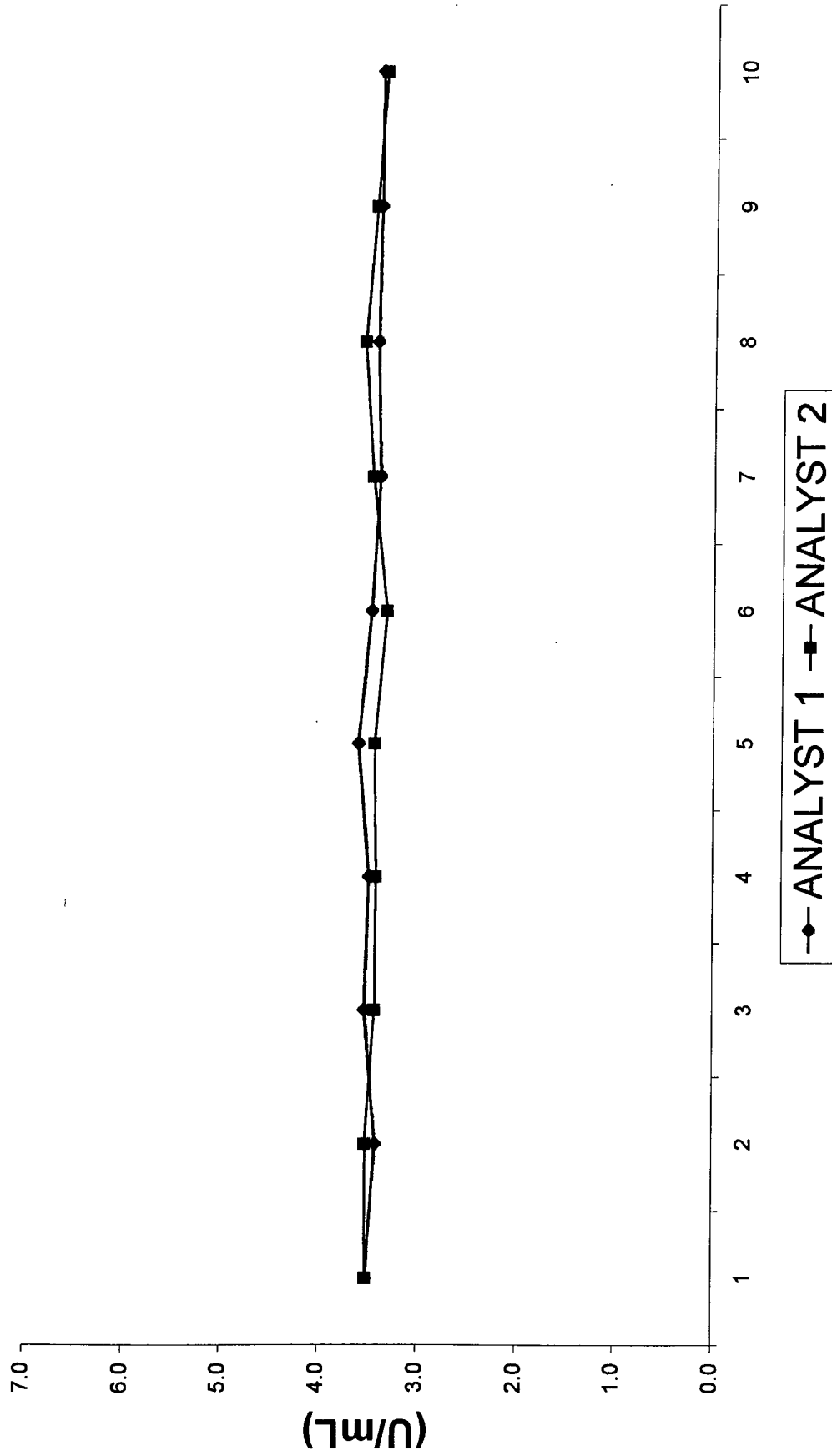
FIGURE 5.

AChE (387-40)
ANALYSES PERFORMED OVER 5 DAY PERIOD

ANALYST 1 — ANALYST 2
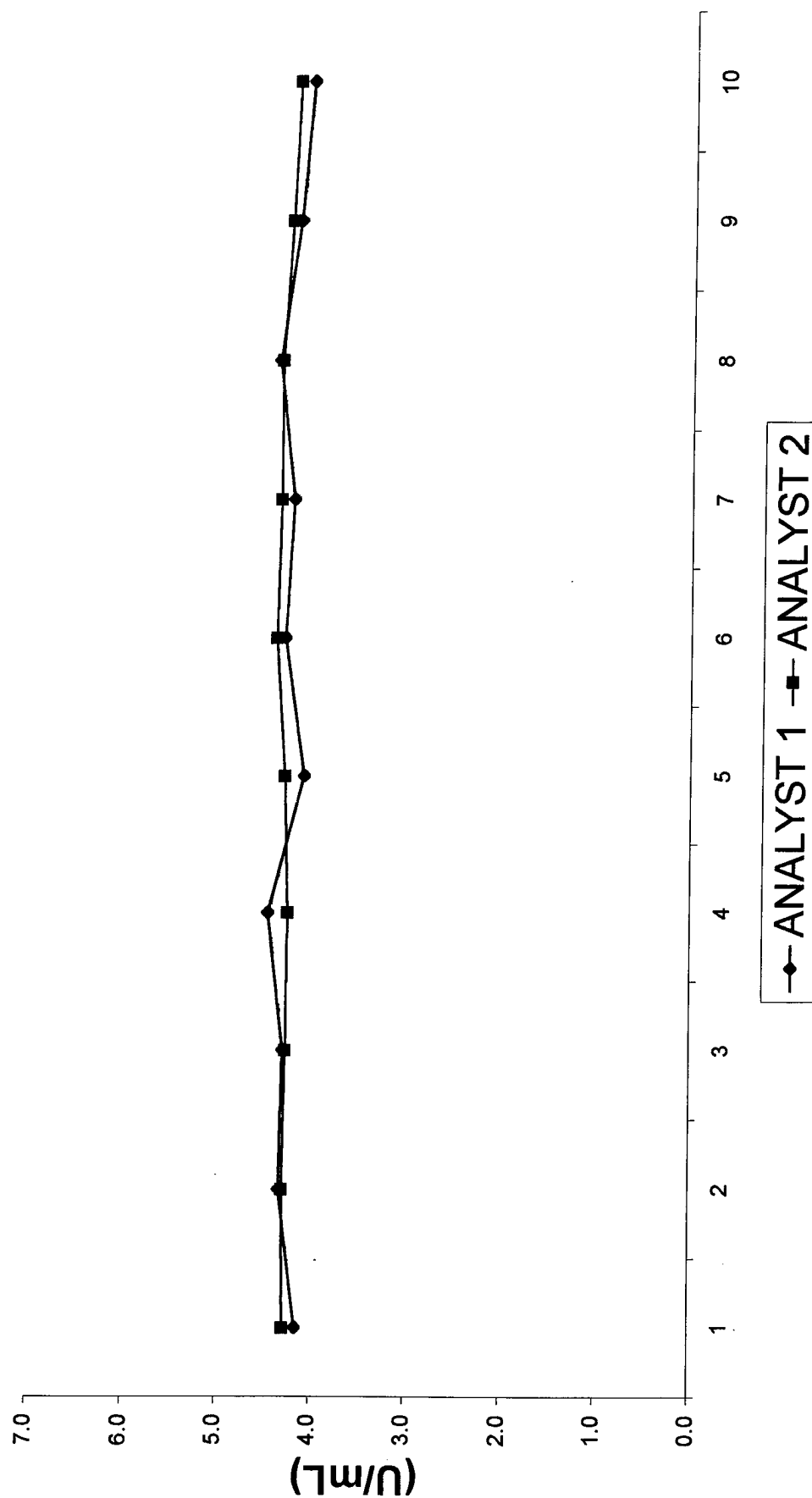
FIGURE 6.

AChE (387-44)
ANALYSES PERFORMED OVER 5 DAY PERIOD

→ ANALYST 1 → ANALYST 2
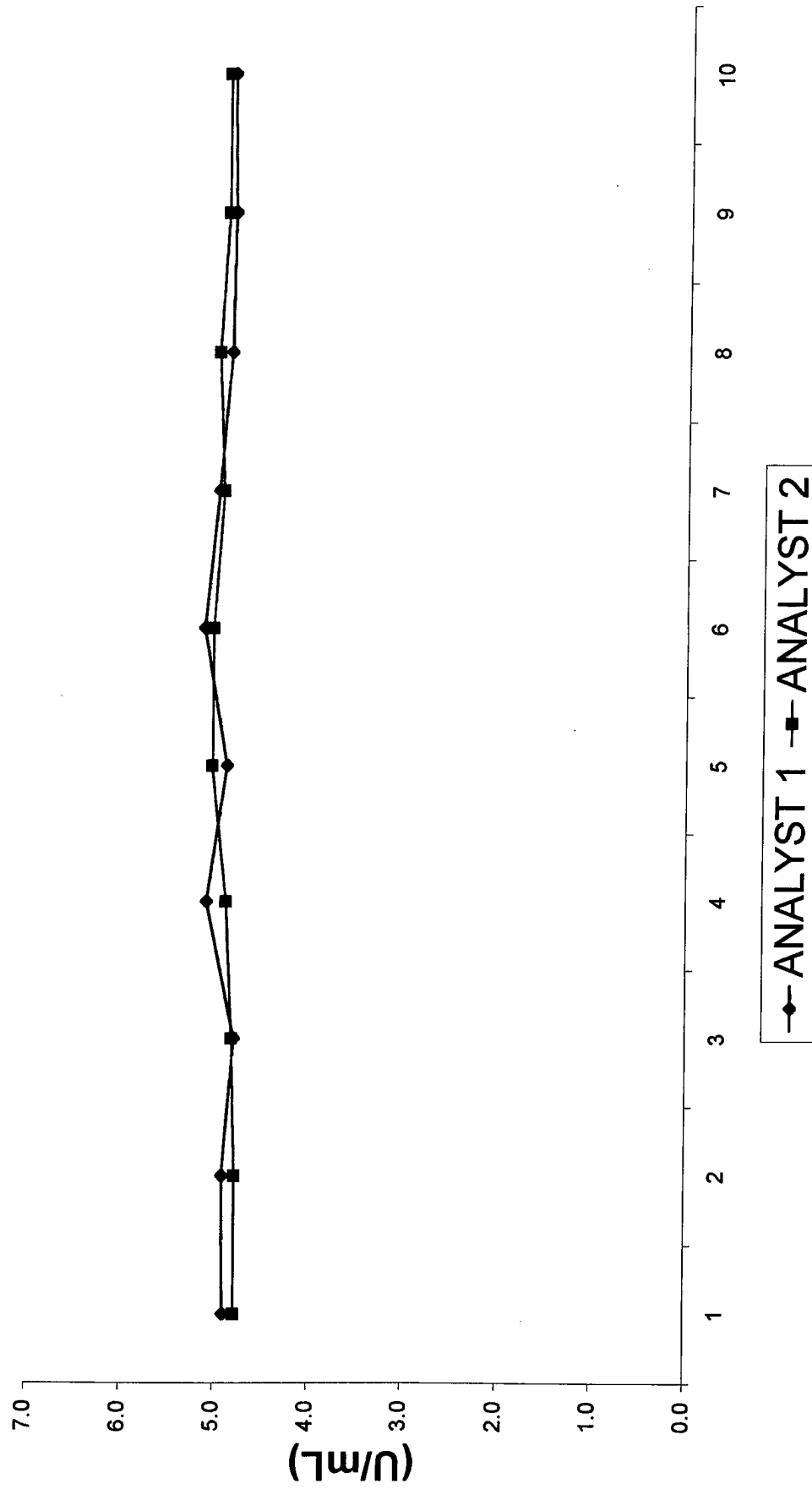
FIGURE 7.

AChE (387-48)
ANALYSES PERFORMED OVER 5 DAY PERIOD

U/mL

—◆— ANALYST 1 —■— ANALYST 2
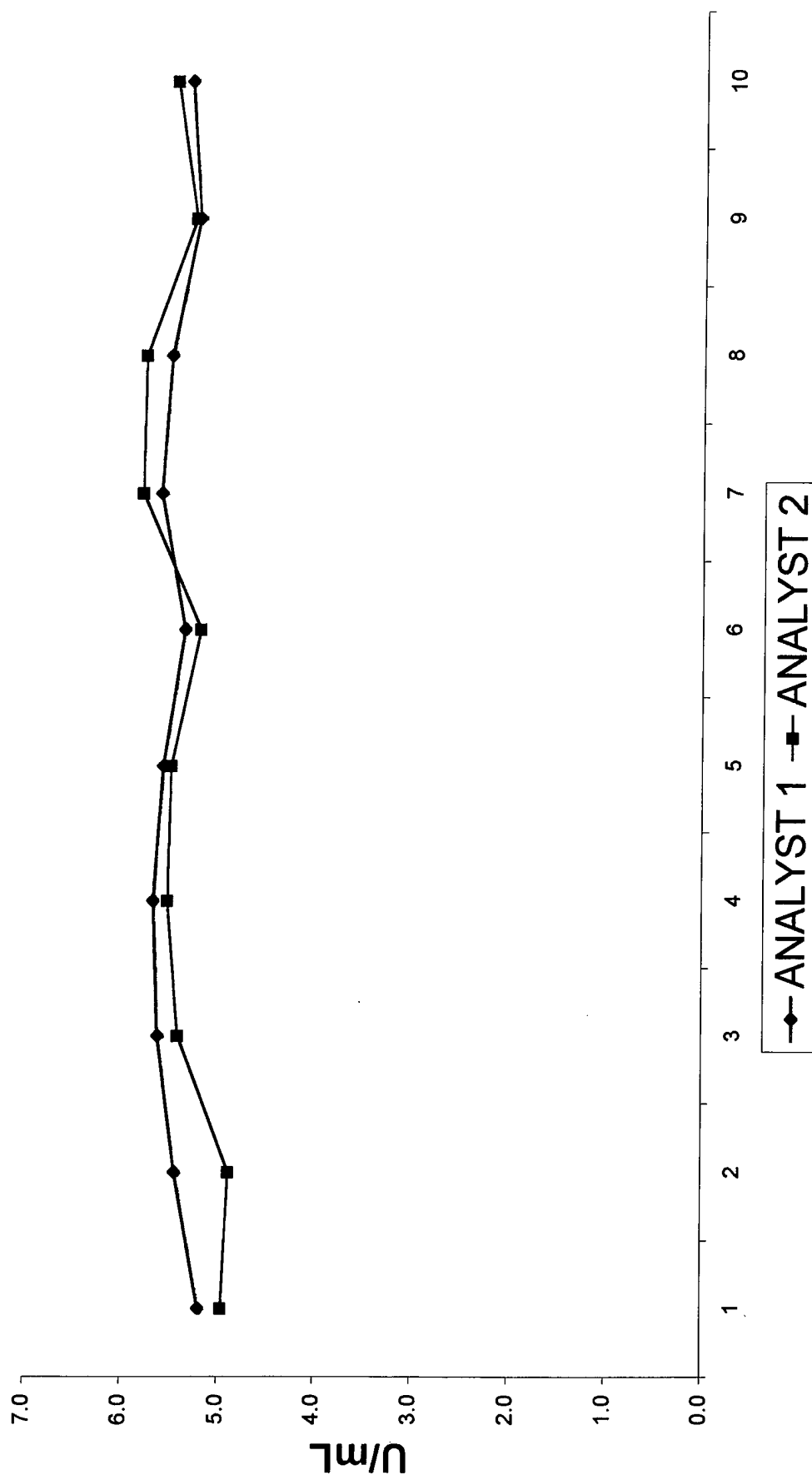
FIGURE 8.

PChE (387-24)
ANALYSES PERFORMED OVER 5 DAY PERIOD
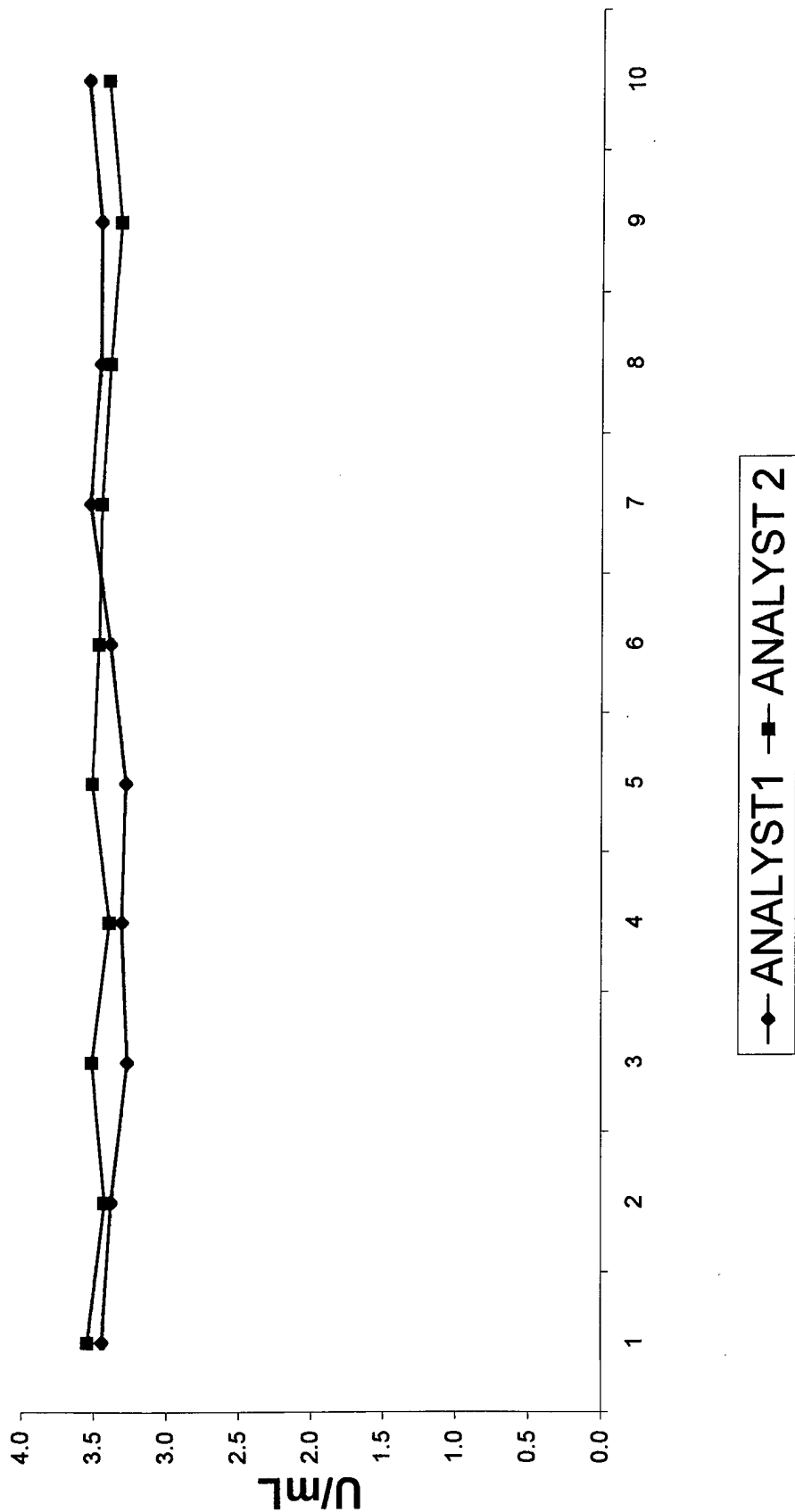
FIGURE 9.

PChE (387-28)
ANALYSES PERFORMED OVER 5 DAY PERIOD
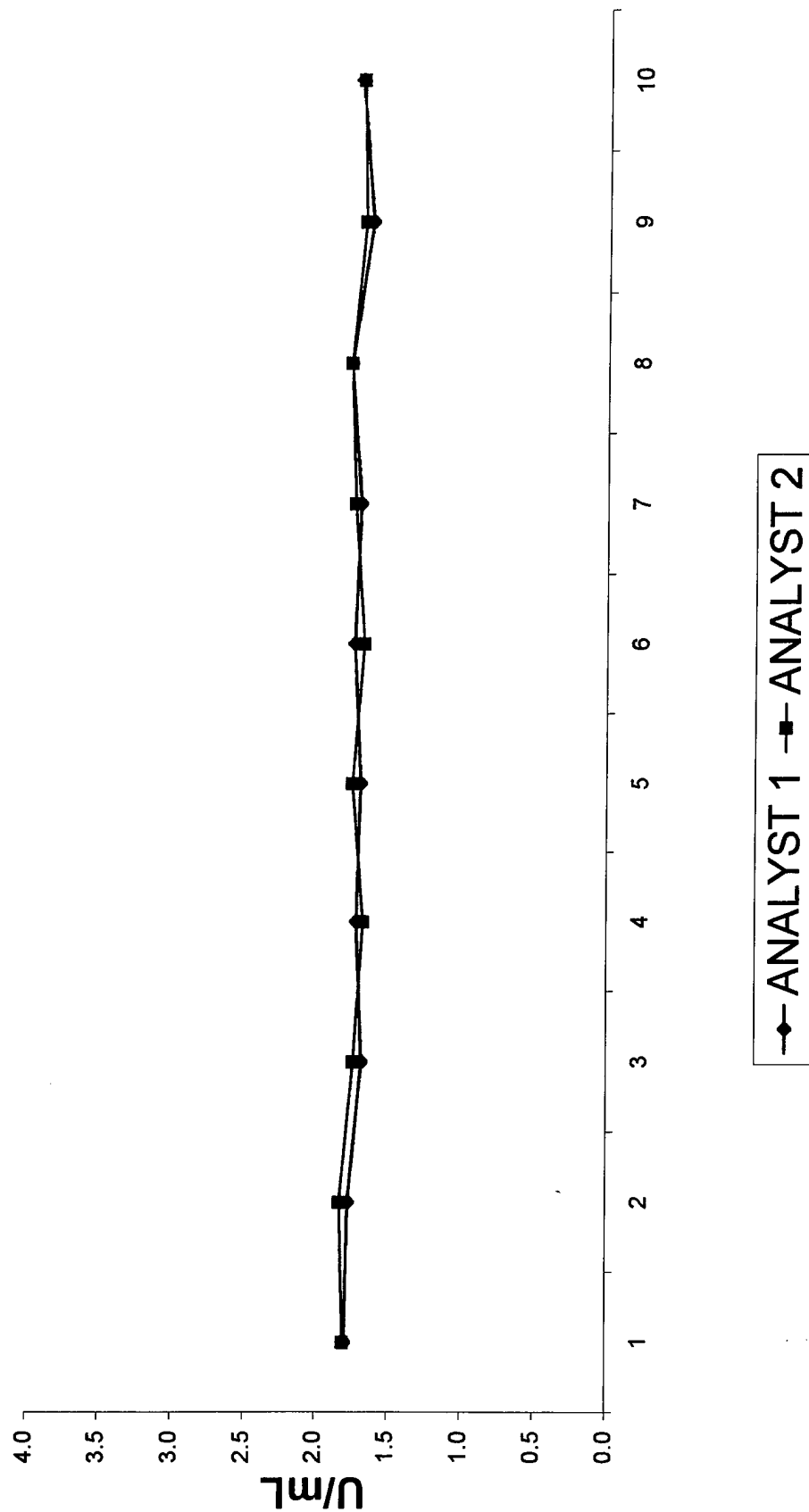
FIGURE 10.

PChE (387-32)
ANALYSES PERFORMED OVER 5 DAY PERIOD

─◆─ ANALYST 1 ─■─ ANALYST 2
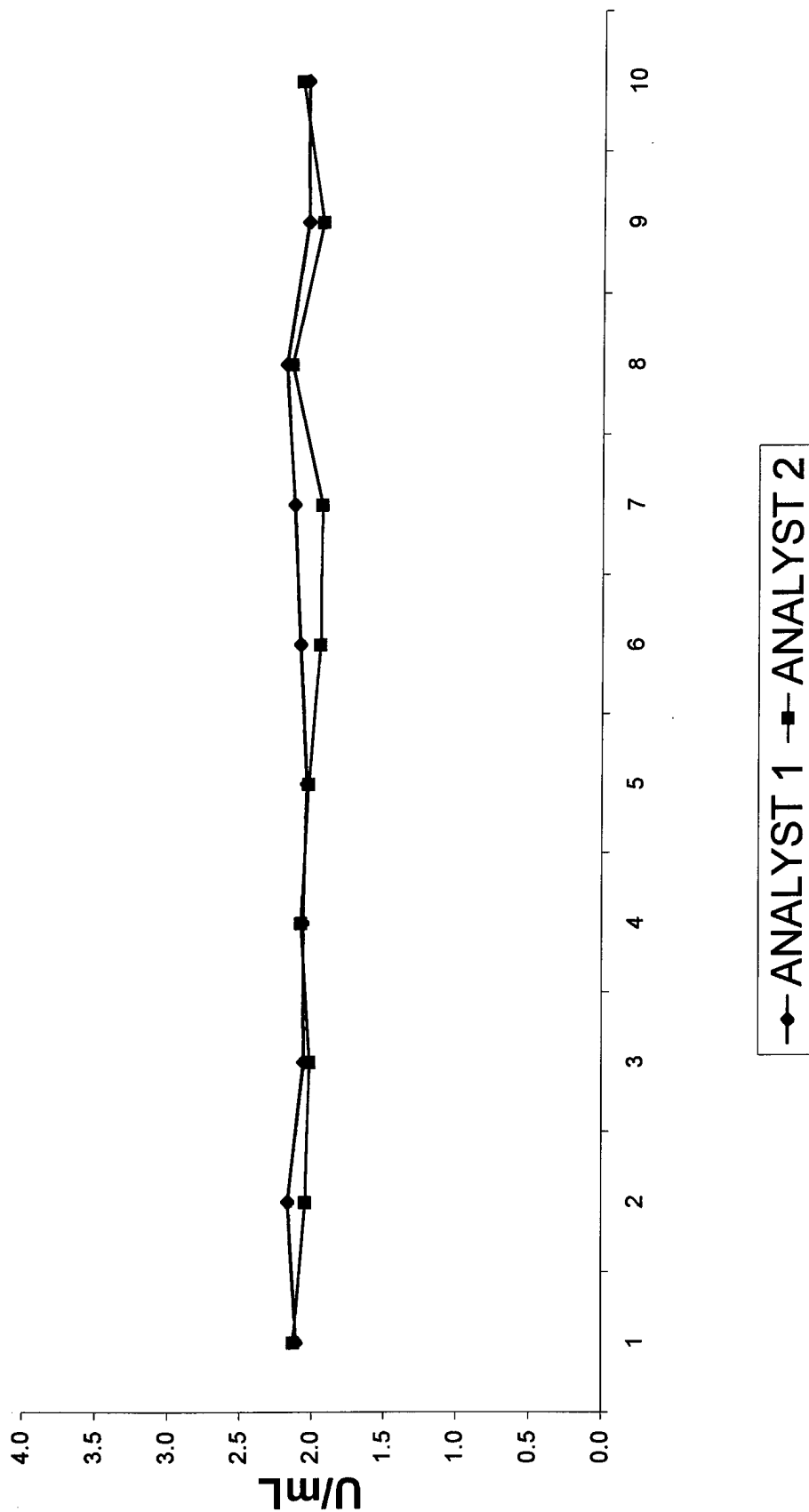
FIGURE 11.

PChE (387-36)
ANALYSES PERFORMED OVER 5 DAY PERIOD

U/mL

ANALYST 1   ANALYST 2
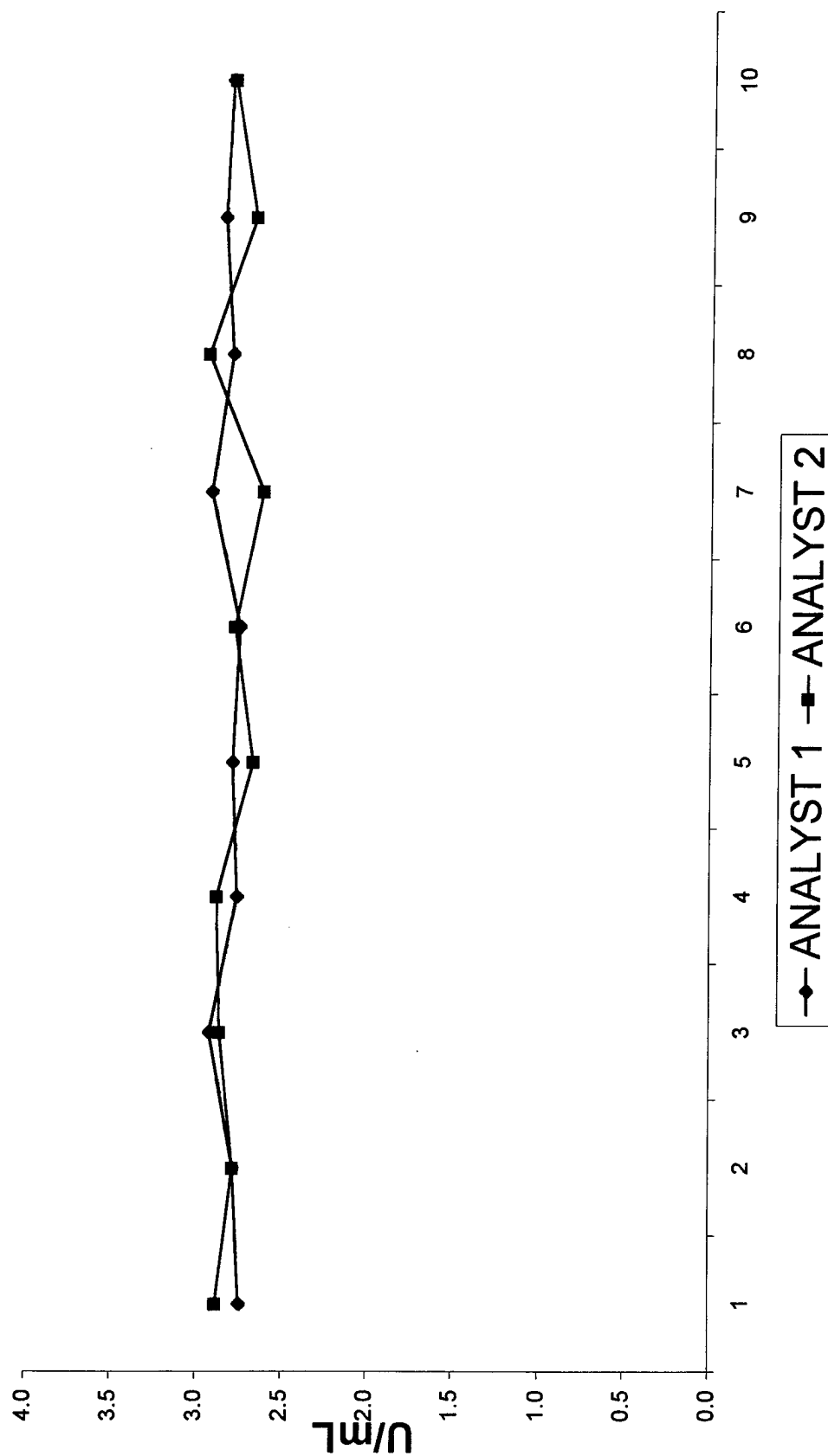
FIGURE 12.

PChE (387-40)
ANALYSES PERFORMED OVER 5 DAY PERIOD
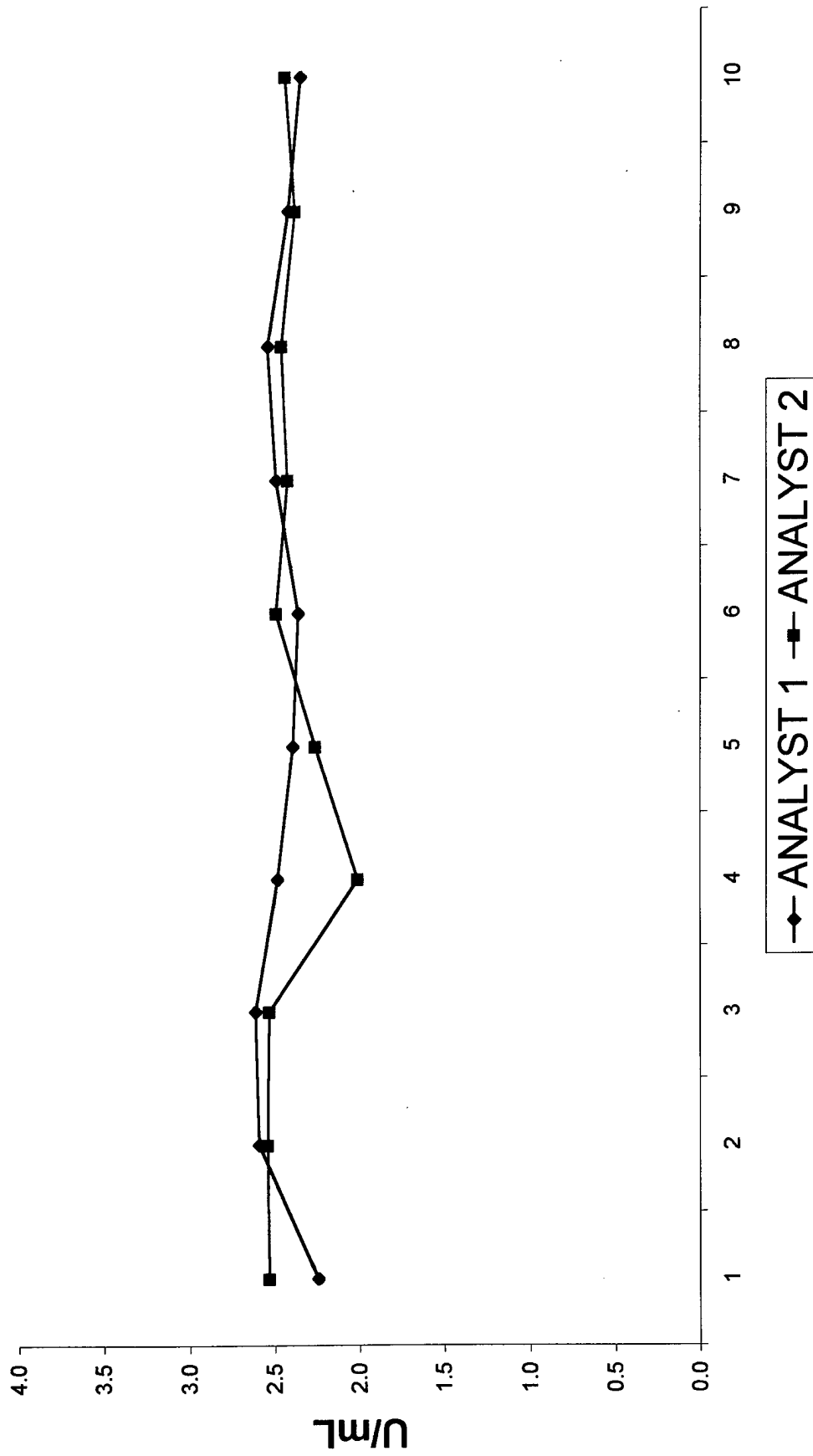
FIGURE 13.

PChE (387-44)
ANALYSES PERFORMED OVER 5 DAY PERIOD
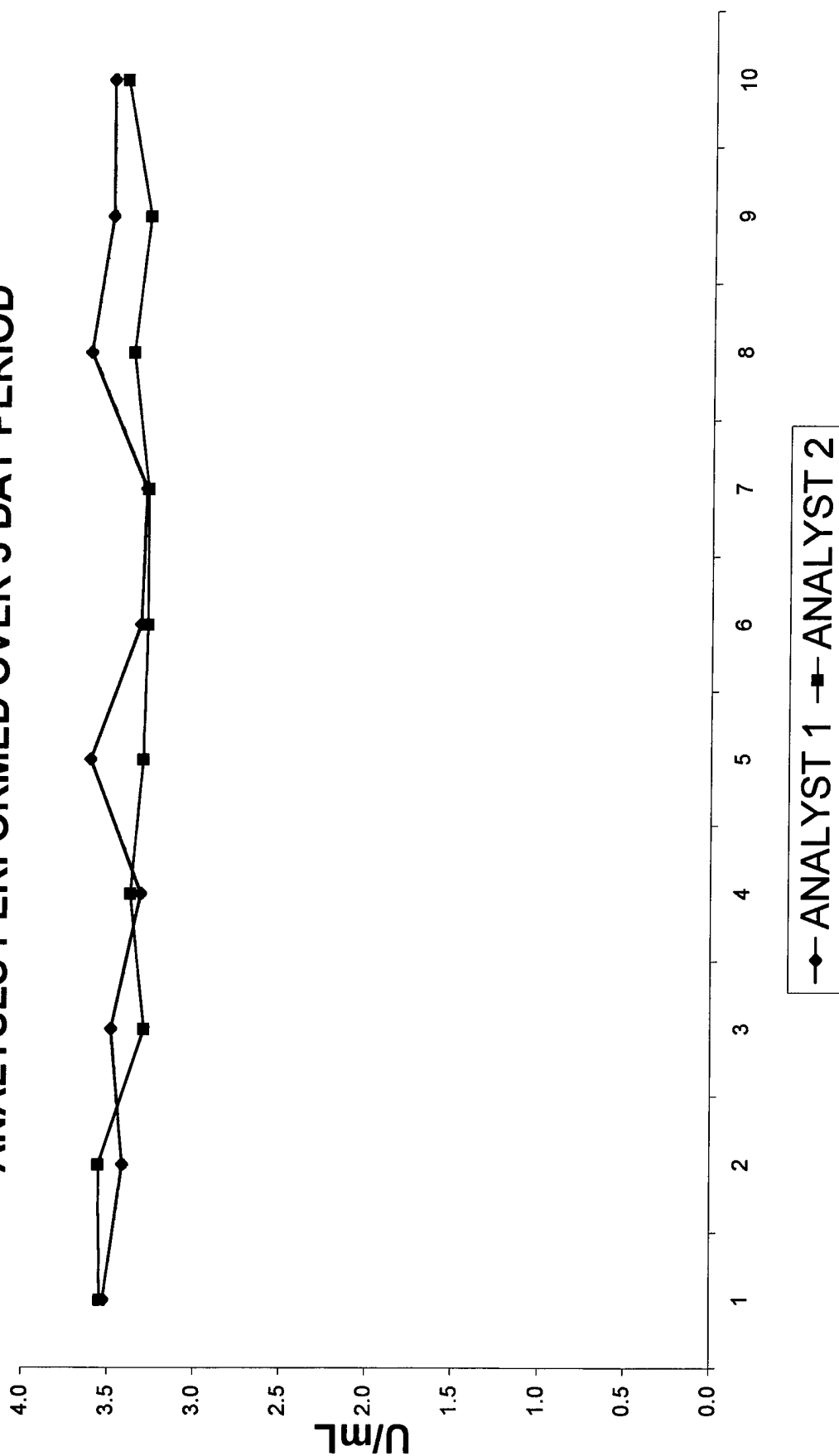
ANALYST 1 — ANALYST 2

FIGURE 14.

PChE (387-48)
ANALYSES PERFORMED OVER 5 DAY PERIOD

Table 17. AChE Linearity Data Sample 387-32

Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
0	8398	1307	1	6.04	128	14.7	98	41.0	131
0	8398	1311	1	6.17	131	14.9	99	41.5	132
0	8398	1317	2	6.11	130	15.1	101	40.4	129
0	8398	1321	2	6.16	131	14.9	99	41.3	132
10	8398	1324	1	3.81	81	14.5	97	26.3	84
10	8398	1328	1	3.84	82	14.9	100	25.7	82
10	8398	1334	2	3.94	84	15.2	101	26.0	83
10	8398	1339	2	3.86	82	14.8	99	26.0	83
20	8398	1341	1	2.25	48	14.9	99	15.1	48
20	8398	1346	1	2.48	53	15.0	100	16.6	53
20	8398	1351	2	2.45	52	14.8	98	16.6	53
20	8398	1356	2	2.46	52	14.6	98	16.8	53
30	8398	1400	1	0.06	1	14.9	99	0.4	1
30	8398	1404	1	0	0	14.8	98	0.0	0
30	8398	1408	2	0	0	14.7	98	0.0	0
30	8398	1414	2	-0.03	0	14.9	99	-0.2	0
40	8398	1418	1	-0.11	-2	14.6	98	-0.8	-2
40	8398	1422	1	-0.10	-2	14.9	100	-0.6	-2
40	8398	1426	2	-0.11	-2	14.5	97	-0.7	-2
40	8398	1431	2	-0.12	-2	14.7	98	-0.8	-3
50	8398	1436	1	-0.18	-4	14.8	99	-1.2	-4
50	8398	1440	1	-0.07	-1	15.1	101	-0.4	-1
50	8398	1445	2	-0.10	-2	14.9	99	-0.7	-2
50	8398	1449	2	-0.36	-8	14.8	98	-2.4	-8

AVG=   14.8

STD=   0.2

%RSD=   1.2%

Table 18. AChE Linearity Data Sample 387-36

Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
0	8498	752	1	3.44	73	12.6	84	27.3	87
0	8498	758	1	3.35	71	12.7	85	26.3	84
0	8498	801	2	3.46	74	12.7	85	27.3	87
0	8498	806	2	3.15	67	12.5	83	25.2	80
10	8498	820	1	2.80	59	12.9	86	21.6	69
10	8498	825	1	2.91	62	12.6	84	23.1	74
10	8498	811	2	2.92	62	12.6	84	23.1	74
10	8498	816	2	2.81	60	12.4	82	22.7	72
20	8498	830	1	2.45	52	12.5	83	19.7	63
20	8498	838	1	2.35	50	12.2	81	19.3	61
20	8498	839	2	2.38	51	12.3	82	19.4	62
20	8498	844	2	2.43	52	12.5	83	19.4	62
30	8498	850	1	1.87	40	12.5	84	14.9	48
30	8498	855	1	1.79	38	12.5	83	14.4	46
30	8498	859	2	1.94	41	12.7	85	15.2	49
30	8498	902	2	1.88	40	12.8	85	14.7	47
40	8498	906	1	1.49	32	12.5	83	12.0	38
40	8498	911	1	1.47	31	12.6	84	11.7	37
40	8498	915	2	1.55	33	12.5	84	12.4	39
40	8498	919	2	1.57	33	12.6	84	12.5	40
50	8498	923	1	1.49	32	12.6	84	11.8	38
50	8498	928	1	1.53	32	12.6	84	12.1	39
50	8498	932	2	1.61	34	12.6	84	12.8	41
50	8498	936	2	1.58	34	12.8	85	12.4	39

AVG=   12.6

STD=   0.2

%RSD=   1.2%

Table 19. AChE Linearity Data Sample 387-40

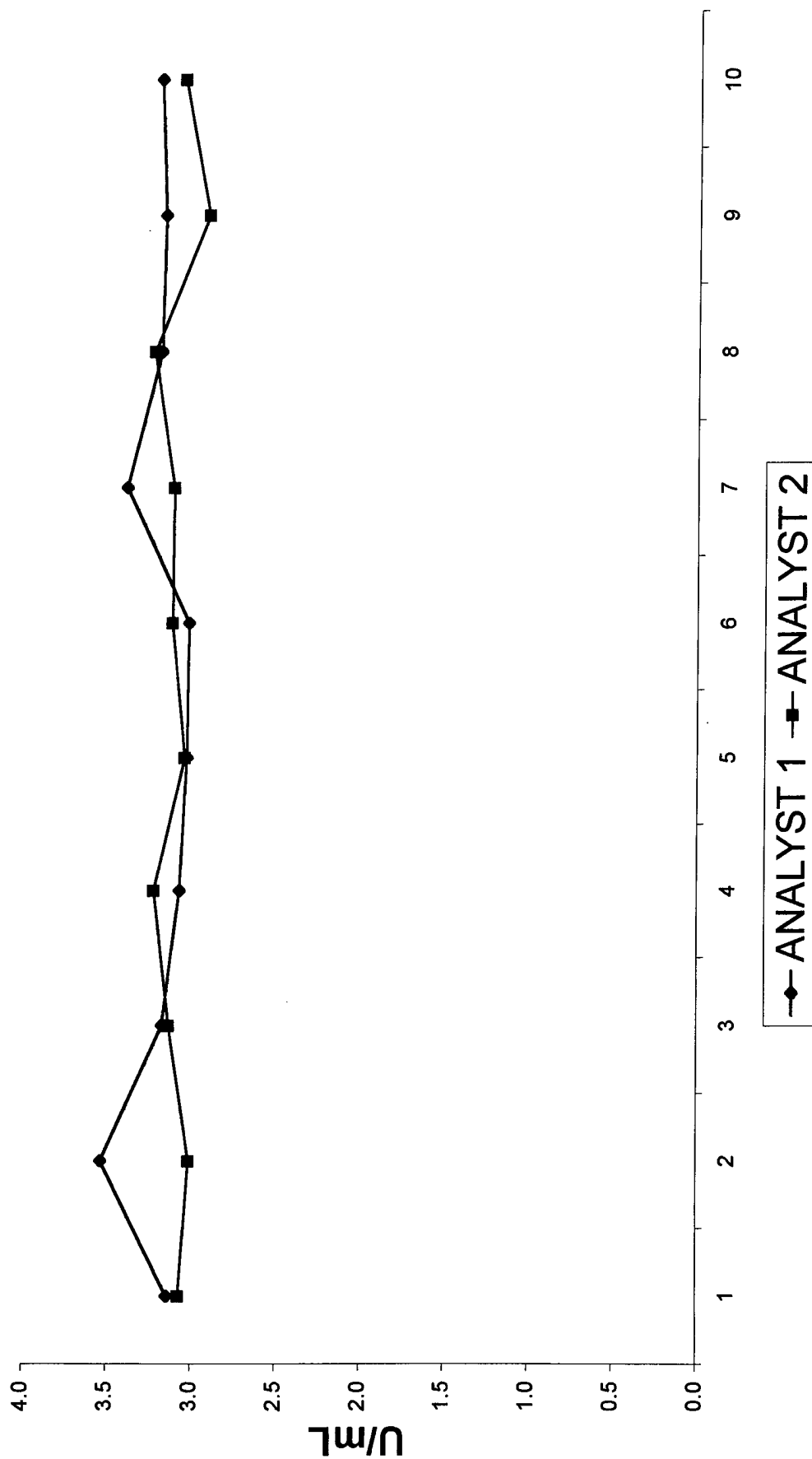| Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
0	8498	1241	1	4.22	90	13.4	90	31.4	100
0	8498	1247	1	4.42	94	13.6	91	32.4	103
0	8498	1232	2	4.29	91	13.9	93	30.9	98
0	8498	1237	2	4.30	91	13.5	90	31.9	102
10	8498	1253	1	3.76	80	13.6	91	27.7	88
10	8498	1258	1	3.70	79	13.5	90	27.5	88
10	8498	1318	2	3.66	78	13.5	90	27.0	86
10	8498	1323	2	3.81	81	14.0	93	27.2	87
20	8498	1308	1	3.33	71	13.6	91	24.4	78
20	8498	1313	1	3.44	73	14.0	93	24.6	78
20	8498	1328	2	3.36	71	13.8	92	24.3	78
20	8498	1331	2	3.22	68	13.6	90	23.8	76
30	8498	1336	1	2.60	55	13.5	90	19.2	61
30	8498	1340	1	2.72	58	13.7	91	19.9	63
30	8498	1344	2	2.83	60	13.8	92	20.4	65
30	8498	1347	2	2.65	56	13.7	92	19.3	61
40	8498	1351	1	2.57	54	13.5	90	19.0	61
40	8498	1356	1	2.54	52	13.5	90	18.2	58
40	8498	1401	2	2.59	55	13.4	89	19.3	62
40	8498	1405	2	2.59	55	13.8	92	18.7	60
50	8498	1410	1	1.90	40	13.6	91	13.9	44
50	8498	1415	1	2.01	43	13.3	88	15.1	48
50	8498	1419	2	2.12	45	13.3	89	15.9	51
50	8498	1422	2	2.04	43	13.8	92	14.8	47

AVG=   13.6
STD=   0.2
%RSD=   1.5%

Table 20. AChE Linearity Data Sample 387-44

Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
0	8598	1423	1	5.04	107	14.4	96	34.9	111
0	8598	1437	1	4.89	104	14.8	99	33.0	105
0	8598	1445	2	4.66	99	14.6	97	31.9	101
0	8598	1451	2	4.99	106	14.7	98	34.0	108
10	8598	1456	1	4.53	96	14.8	99	30.6	97
10	8598	1504	1	4.75	101	14.6	98	32.5	103
10	8598	1510	2	4.65	99	14.7	98	31.7	101
10	8598	1514	2	4.45	94	14.5	97	30.6	97
20	8598	1517	1	4.11	87	14.6	97	28.1	90
20	8598	1521	1	4.28	91	14.6	97	29.4	94
20	8598	1526	2	4.11	87	14.7	98	27.9	89
20	8598	1531	2	4.28	87	14.7	98	27.8	88
30	8598	1536	1	3.78	80	14.2	95	26.6	85
30	8598	1540	1	3.78	80	14.5	97	26.1	83
30	8598	1554	2	3.79	80	15.0	100	25.3	81
30	8598	1559	2	3.90	83	14.7	98	26.5	84
40	8598	1546	1	3.75	80	14.5	96	26.0	83
40	8598	1552	1	3.64	77	14.9	99	24.4	78
40	8598	1603	2	3.64	77	15.0	100	24.3	78
40	8598	1606	2	3.71	79	14.7	98	25.2	80
50	8598	1617	1	3.11	66	14.5	96	21.5	69
50	8598	1621	1	3.09	66	15.7	104	19.8	63
50	8598	1610	2	3.22	68	14.8	98	21.8	69
50	8598	1614	2	3.09	66	14.9	99	20.8	66

AVG=   14.7

STD=   0.3

%RSD=   1.9%

Table 21. AChE Linearity Data Sample 387-48

Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	AChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal	Q (U/g)	Percent of Normal
0	8698	1010	1	5.39	115	13.8	92	39.1	124
0	8698	1014	1	5.51	117	14.1	94	39.1	125
0	8698	1020	2	5.68	121	14.3	95	39.7	126
0	8698	1024	2	5.74	122	14.3	96	40.0	127
10	8698	1028	1	4.98	106	14.2	95	35.0	111
10	8698	1034	1	4.88	104	13.8	92	35.4	115
10	8698	1037	2	5.11	108	13.8	92	36.9	118
10	8698	1041	2	5.16	109	14.2	95	36.3	116
20	8698	1046	1	4.61	98	14.3	96	32.1	102
20	8698	1051	1	4.76	101	14.0	93	34.1	109
20	8698	1056	2	4.77	101	13.9	92	34.4	110
20	8698	1100	2	4.73	100	14.0	94	33.7	107
30	8698	1105	1	4.22	90	14.1	94	29.9	95
30	8698	1110	1	4.32	92	14.3	95	30.2	96
30	8698	1114	2	4.40	93	13.8	92	31.8	101
30	8698	1118	2	4.33	92	13.8	92	31.4	100
40	8698	1133	1	4.10	87	13.9	93	29.4	94
40	8698	1126	1	4.15	88	14.0	93	29.7	95
40	8698	1137	2	4.07	86	13.8	92	29.5	94
40	8698	1143	2	4.14	88	14.4	96	28.8	92
50	8698	1146	1	3.54	75	14.2	95	24.9	79
50	8698	1151	1	3.52	75	14.2	95	24.7	79
50	8698	1156	2	3.44	73	14.1	94	24.5	78
50	8698	1200	2	3.56	76	14.3	95	24.9	79

AVG=   14.1

STD=   0.2

%RSD=   1.4%

Table 22. PChE Linearity Data Sample 387-32

Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
0	8398	1503	1	2.13	84	14.7	98
0	8398	1507	1	2.09	82	14.8	99
0	8398	1454	2	2.02	79	15.1	101
0	8398	1459	2	2.02	79	15.3	102
10	8398	1512	1	1.41	55	15.0	100
10	8398	1516	1	1.30	51	14.9	99
10	8398	1520	2	1.43	56	15.0	100
10	8398	1524	2	1.42	56	14.8	99
20	8398	1528	1	0.98	38	14.8	99
20	8398	1532	1	0.92	36	15.1	101
20	8398	1536	2	0.97	38	15.0	100
20	8398	1541	2	0.97	38	14.9	99
30	8398	1545	1	0.19	8	14.6	97
30	8398	1549	1	0.32	12	14.7	98
30	8398	1553	2	0.36	14	14.6	97
30	8398	1556	2	0.28	11	14.5	96
40	8398	1600	1	0.00	0	14.8	99
40	8398	1604	1	-0.03	-1	14.7	98
40	8398	1609	2	-0.04	-1	14.9	99
40	8398	1613	2	0.06	2	14.9	99
50	8398	1621	1	0.00	0	14.7	98
50	8398	1625	1	-0.02	0	14.8	99
50	8398	1616	2	0.04	1	14.9	99
50	8398	1619	2	0.06	2	15.2	101

AVG=   14.9

STD=   0.2

%RSD=   1.3%

Table 23. PChE Linearity Data Sample 387-36

Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
0	8498	1016	1	2.87	113	12.6	84
0	8498	1026	1	2.81	110	12.3	82
0	8498	1031	2	2.91	114	13.0	86
0	8498	1036	2	3.15	124	12.7	85
10	8498	1040	1	2.46	96	12.4	82
10	8498	1045	1	2.43	95	12.6	84
10	8498	1049	2	2.65	104	13.2	88
10	8498	1053	2	2.54	100	12.9	86
20	8498	1056	1	2.06	81	12.5	83
20	8498	1100	1	2.19	86	12.5	84
20	8498	1104	2	2.18	86	12.8	86
20	8498	1108	2	2.16	85	12.5	83
30	8498	1111	1	2.02	79	12.6	84
30	8498	1115	1	1.89	74	12.7	85
30	8498	1120	2	1.90	75	12.8	85
30	8498	1124	2	1.65	65	12.5	83
40	8498	1127	1	1.70	67	12.5	83
40	8498	1130	1	1.59	62	12.7	85
40	8498	1135	2	1.68	66	12.6	84
40	8498	1139	2	1.67	65	12.6	84
50	8498	1144	1	1.36	53	12.4	83
50	8498	1147	1	1.35	53	12.6	84
50	8498	1151	2	1.27	50	12.4	82
50	8498	1156	2	1.37	54	12.9	86

AVG=   12.6
STD=   0.2
%RSD=   1.7%

Table 24. PChE Linearity Data Sample 387-40

Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
0	8498	1427	1	2.55	100	13.5	90
0	8498	1429	1	2.56	100	13.6	91
0	8498	1435	2	2.42	95	13.9	92
0	8498	1440	2	2.60	102	13.7	91
10	8498	1450	1	2.36	93	13.7	91
10	8498	1503	1	2.22	87	13.8	92
10	8498	1452	2	2.00	79	13.3	89
10	8498	1456	2	2.33	91	13.7	92
20	8498	1500	1	2.10	82	13.3	89
20	8498	1507	1	2.09	82	13.5	90
20	8498	1512	2	2.03	80	13.6	91
20	8498	1516	2	1.99	78	13.5	90
30	8498	1520	1	1.92	75	13.8	92
30	8498	1524	1	1.90	75	13.7	91
30	8498	1528	2	1.79	70	13.6	91
30	8498	1532	2	1.82	71	13.8	92
40	8498	1536	1	1.67	66	13.5	90
40	8498	1540	1	1.70	67	13.7	91
40	8498	1544	2	1.63	64	13.5	90
40	8498	1548	2	1.66	65	13.7	91
50	8498	1607	1	1.40	55	13.5	90
50	8498	1556	1	1.49	58	13.8	92
50	8498	1600	2	1.39	54	13.3	88
50	8498	1604	2	1.45	57	13.6	91

AVG=   13.6

STD=   0.2

%RSD=   1.5%

Table 25. PChE Linearity Data Sample 387-44

Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
0	8698	758	1	3.37	132	14.8	99
0	8698	803	1	3.52	138	14.9	100
0	8698	809	2	3.48	137	15.1	101
0	8698	815	2	3.41	134	15.0	100
10	8698	819	1	3.23	127	14.7	98
10	8698	825	1	3.20	125	14.7	98
10	8698	830	2	3.20	126	14.5	97
10	8698	836	2	3.32	130	14.9	99
20	8698	840	1	2.97	116	14.9	99
20	8698	845	1	2.96	116	14.7	98
20	8698	850	2	2.83	111	14.7	98
20	8698	854	2	2.92	115	14.3	95
30	8698	908	1	2.83	111	14.7	98
30	8698	913	1	2.76	108	14.9	99
30	8698	859	2	2.80	110	14.7	98
30	8698	903	2	2.86	112	15.0	100
40	8698	917	1	2.60	102	14.5	96
40	8698	922	1	2.57	101	14.7	98
40	8698	927	2	2.57	101	14.3	95
40	8698	931	2	2.38	93	14.9	99
50	8698	936	1	2.44	96	14.8	99
50	8698	941	1	2.34	92	15.0	100
50	8698	945	2	2.37	93	15.1	101
50	8698	949	2	2.31	91	15.1	101

AVG=   14.8
STD=   0.2
%RSD=   1.5%

Table 26. PChE Linearity Data Sample 387-48

Amount of Inhibited Blood (μL)	Date of Analysis	Time of Analysis	Analyst Number	PChE (U/mL)	Percent of Normal	Hgb (g/dL)	Percent of Normal
0	8698	1315	1	3.21	126	14.0	94
0	8698	1320	1	3.15	123	14.0	94
0	8698	1325	2	3.10	121	14.1	94
0	8698	1330	2	3.22	126	14.1	94
10	8698	1335	1	2.97	116	14.0	93
10	8698	1339	1	2.97	116	14.3	95
10	8698	1345	2	2.85	112	13.7	91
10	8698	1350	2	2.74	108	14.2	95
20	8698	1354	1	2.74	107	14.1	94
20	8698	1400	1	2.66	104	13.7	91
20	8698	1404	2	2.67	105	14.3	96
20	8698	1409	2	2.72	107	13.9	93
30	8698	1413	1	2.62	103	14.0	93
30	8698	1417	1	2.72	107	14.0	94
30	8698	1421	2	2.74	108	13.7	91
30	8698	1445	2	2.68	105	14.2	95
40	8698	1436	1	2.46	96	14.1	94
40	8698	1441	1	2.46	97	14.2	94
40	8698	1450	2	2.30	90	14.1	94
40	8698	1455	2	2.44	96	14.1	94
50	8698	1458	1	2.11	83	14.2	95
50	8698	1503	1	2.07	81	14.0	94
50	8698	1507	2	2.01	79	14.2	94
50	8698	1512	2	2.06	81	14.2	94

AVG=   14.1

STD=   0.2

%RSD=   1.2%

FIGURE 15.

AChE (387-32)
PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS

ANALYST 1 R^2 = 0.9818
ANALYST 2 R^2 = 0.9845

uL OF INHIBITED RED BLOOD CELLS

(U/mL)

◆ ANALYST 1   ■ ANALYST 2   ——Linear (ANALYST 1)   ——Linear (ANALYST 2)
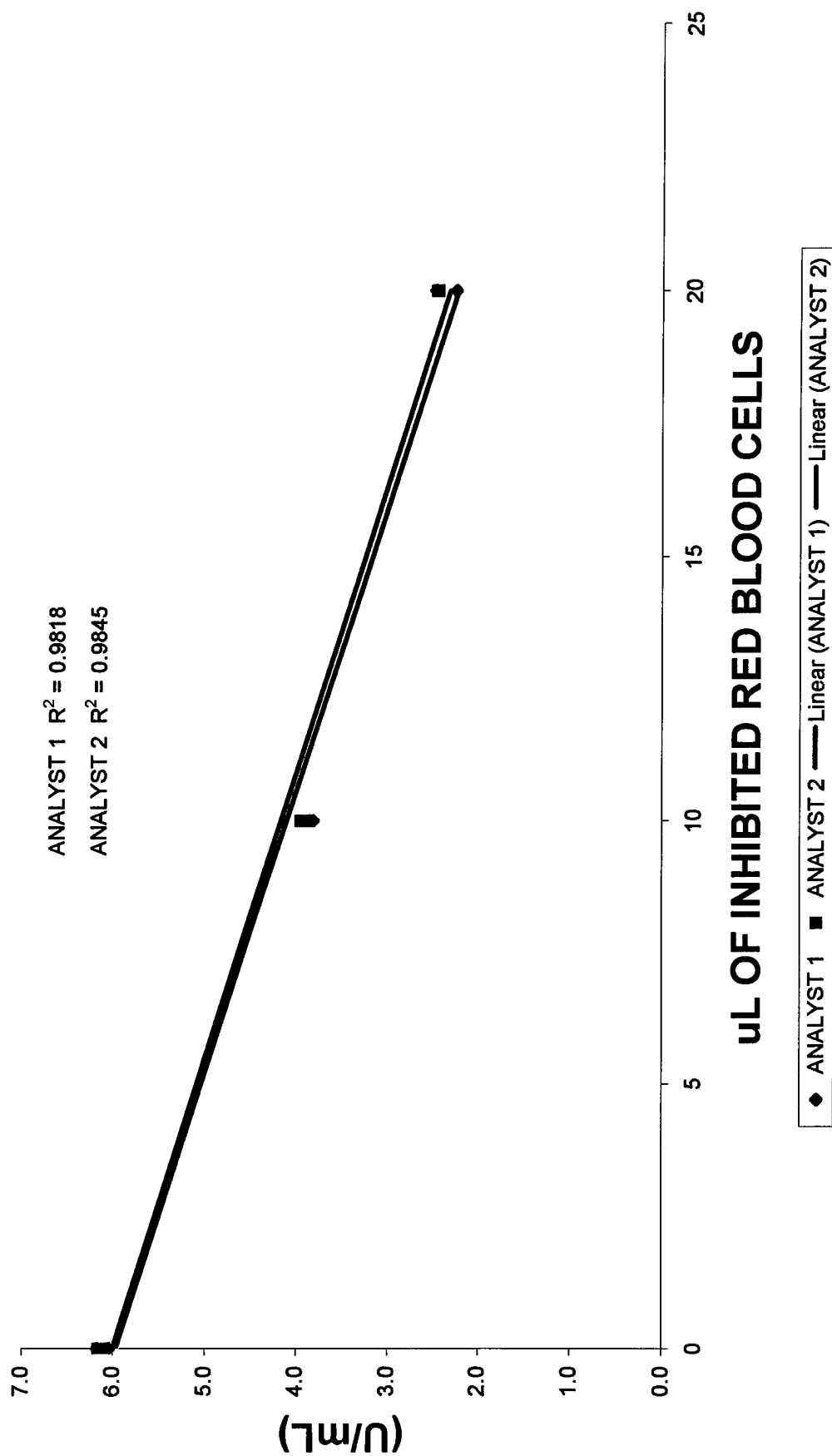
FIGURE 16.

AChE (387-36)

PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS

ANALYST 1 R² = 0.9413
ANALYST 2 R² = 0.9367

uL OF INHIBITED RED BLOOD CELLS

(U/mL)

● ANALYST 1 ■ ANALYST 2 ——Linear (ANALYST 1) ——Linear (ANALYST 2)
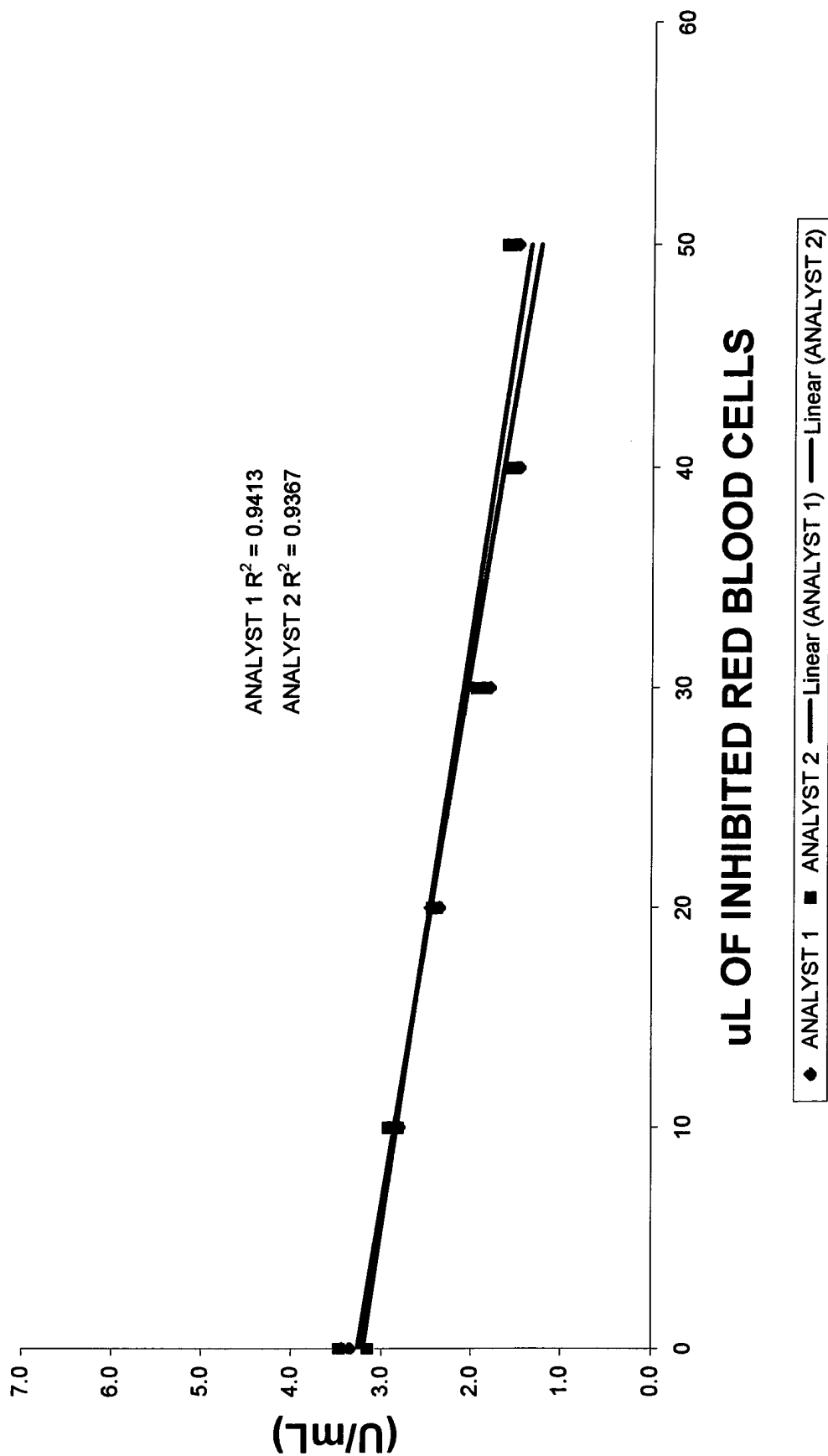
FIGURE 17.

AChE (387-40)

PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS

ANALYST 1 R² = 0.9743
ANALYST 2 R² = 0.9774

uL OF INHIBITED RED BLOOD CELLS

(U/mL)

◆ ANALYST 1   ■ ANALYST 2 ——Linear (ANALYST 1) ——Linear (ANALYST 2)
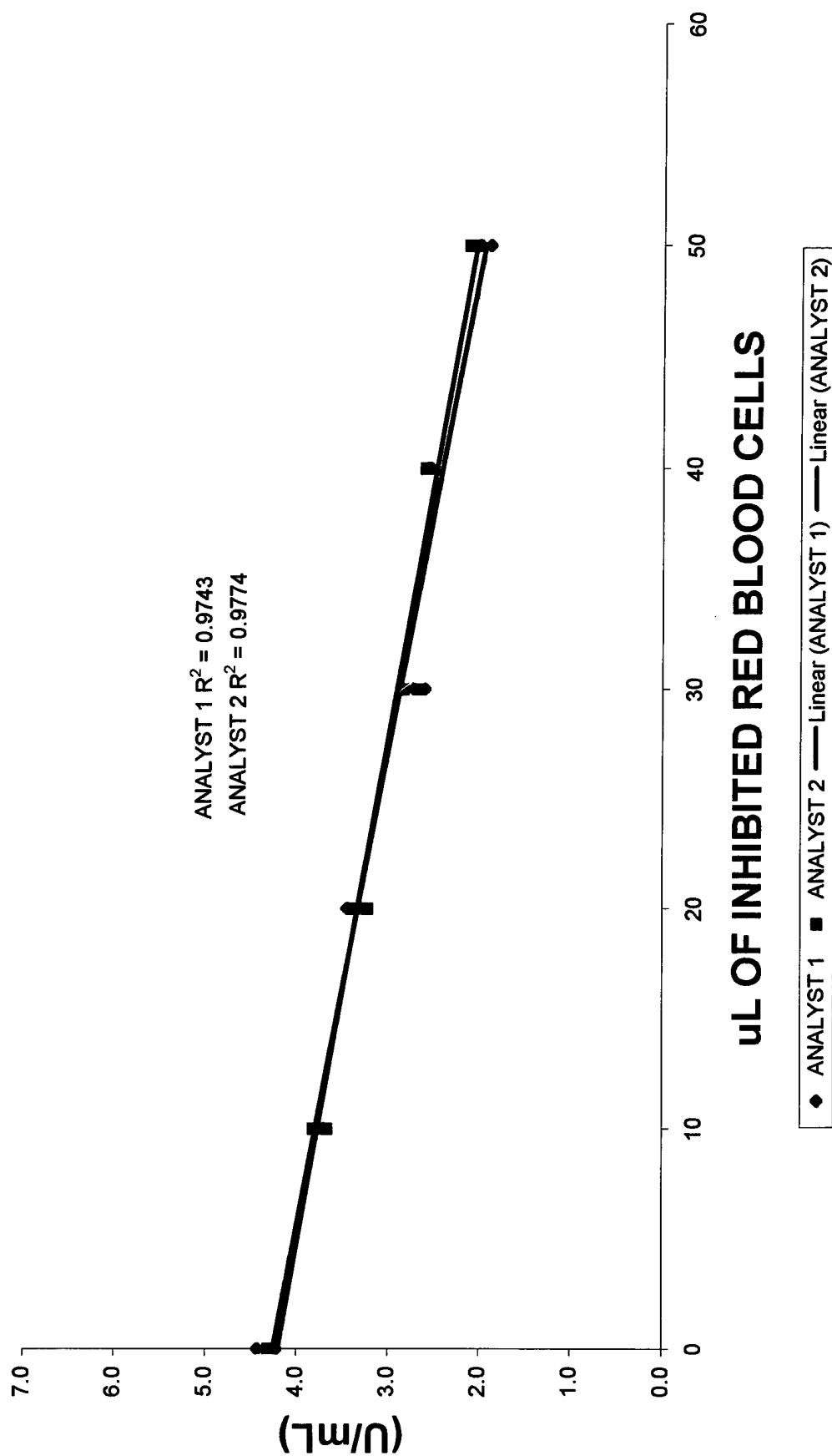
FIGURE 18.

AChE (387-44)

PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS

ANALYST 1 R^2 = 0.9675

ANALYST 2 R^2 = 0.9604

uL OF INHIBITED RED BLOOD CELLS

(U/mL)

◆ ANALYST 1 ■ ANALYST 2 ━━ Linear (ANALYST 1) ━━ Linear (ANALYST 2)
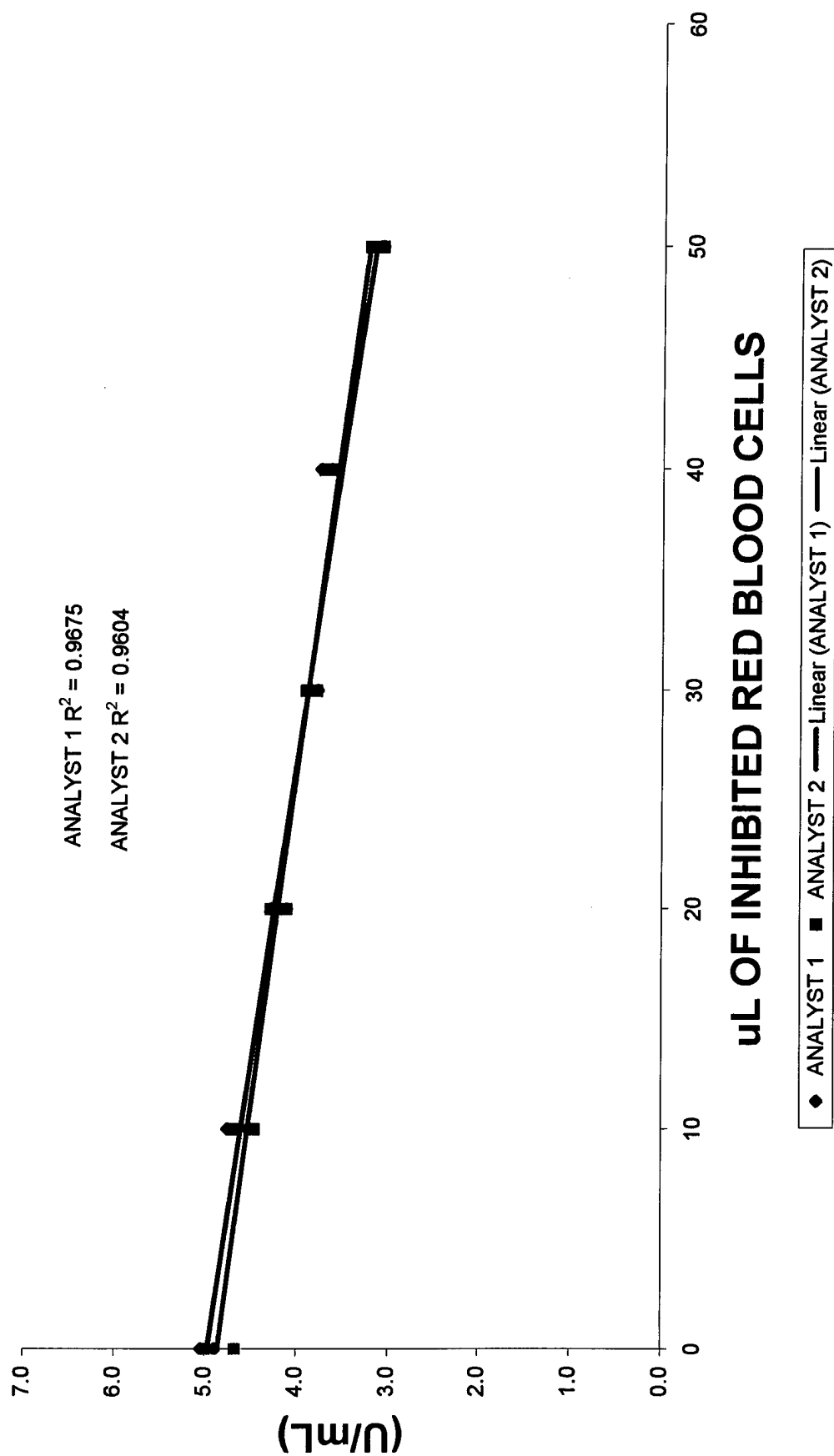
FIGURE 19.

AChE (387-48)
PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS

ANALYST 1 R^2 = 0.9719

ANALYST 2 R^2 = 0.9853
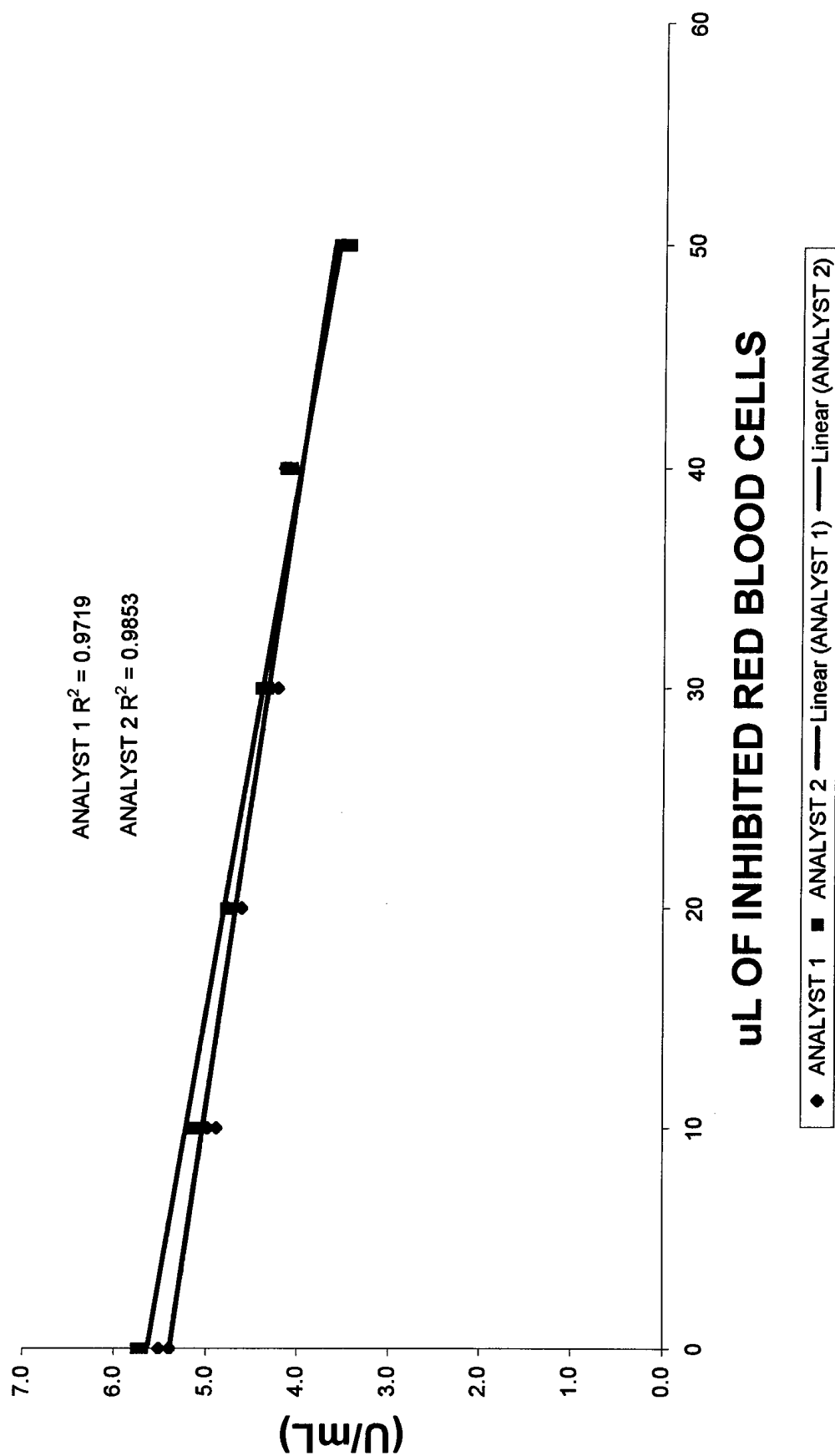
FIGURE 20.

PChE (387-32)
PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS

ANALYST 1 R^2 = 0.9835

ANALYST 2 R^2 = 0.9948

uL OF INHIBITED RED BLOOD CELLS

(U/mL)

♦ ANALYST 1   ■ ANALYST 2   —— Linear (ANALYST 1)   —— Linear (ANALYST 2)
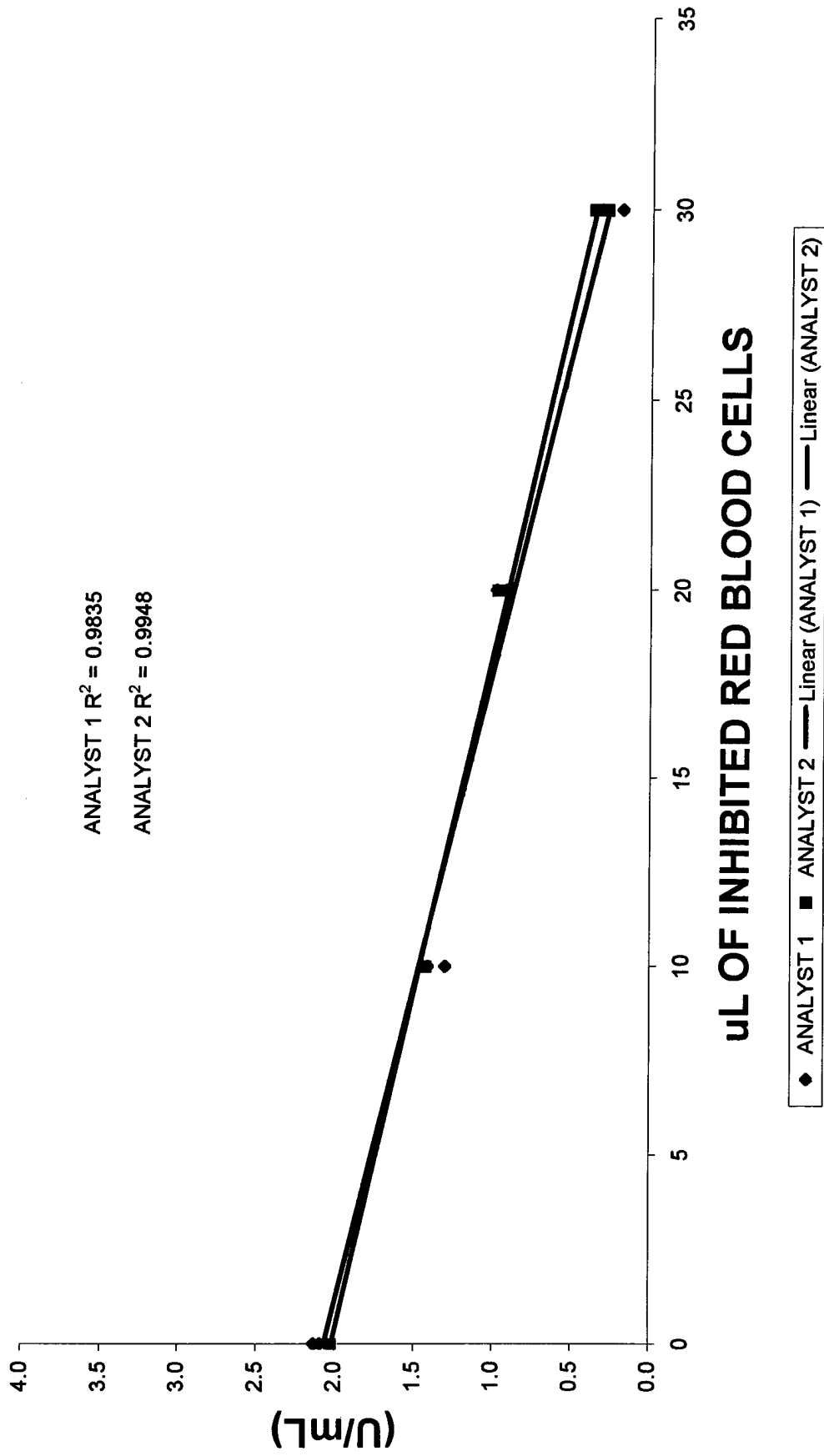
FIGURE 21.

PChE (387-36)
PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS

ANALYST 1 R^2 = 0.9817
ANALYST 2 R^2 = 0.9571

uL OF INHIBITED RED BLOOD CELLS

(U/mL)

♦ ANALYST 1 ■ ANALYST 2 ——Linear (ANALYST 1) ——Linear (ANALYST 2)
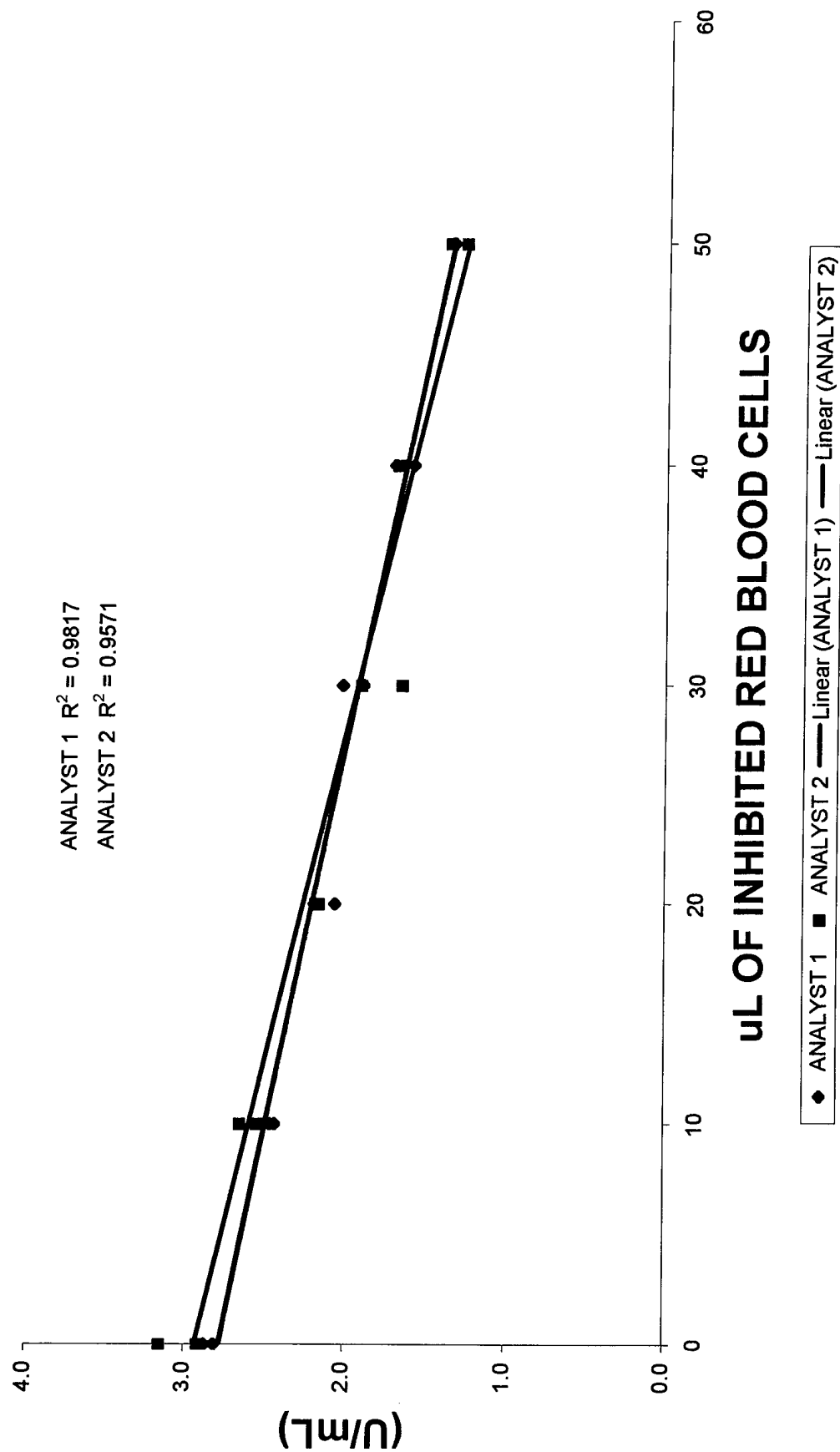
FIGURE 22.

PChE (387-40)
PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS

ANALYST 1 R^2 = 0.9886
ANALYST 2 R^2 = 0.939

◆ ANALYST 1   ■ ANALYST 2 —— Linear (ANALYST 1) —— Linear (ANALYST 2)

uL OF INHIBITED RED BLOOD CELLS

(U/mL)
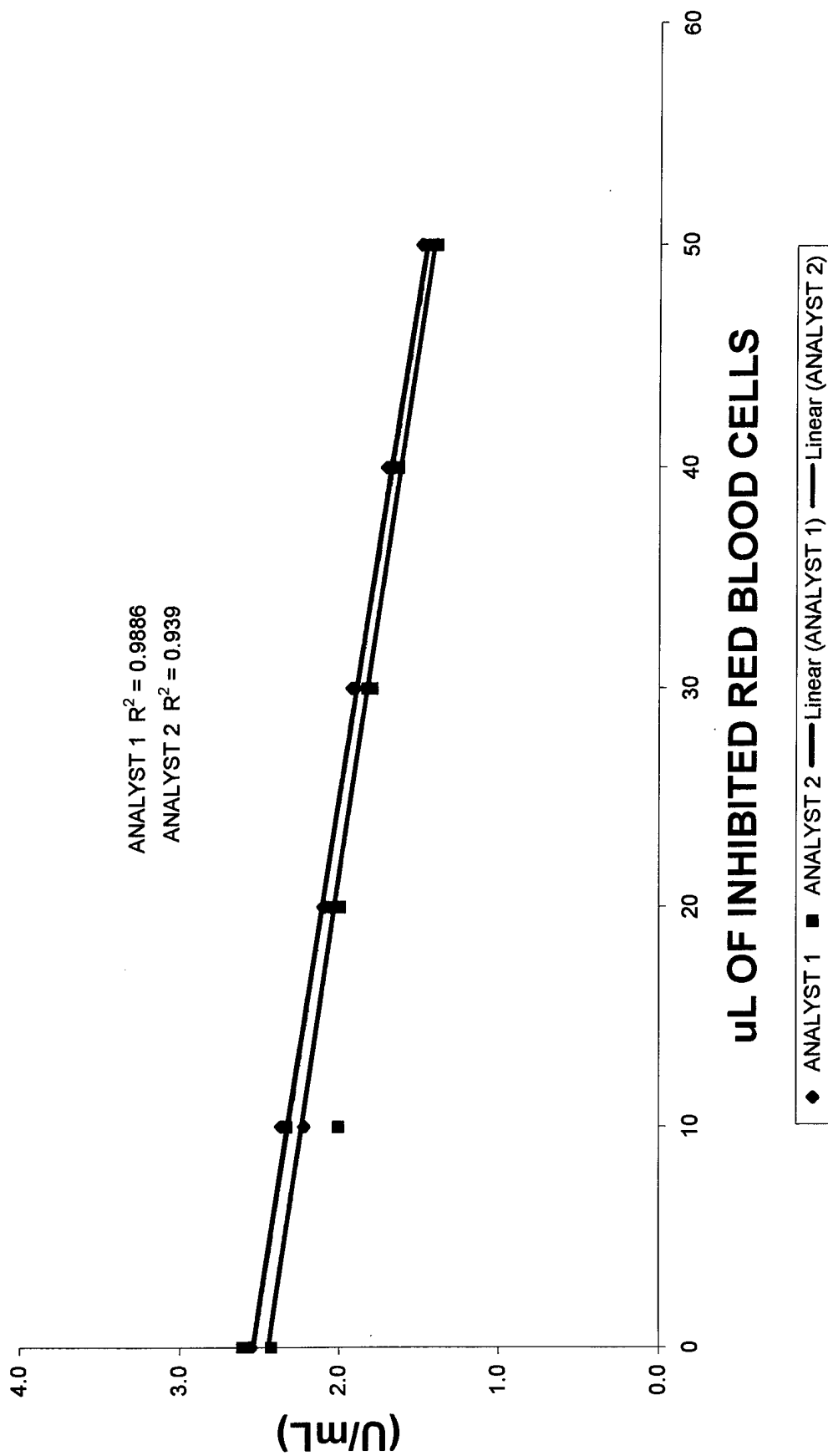
FIGURE 23.

PChE (387-44)

PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS
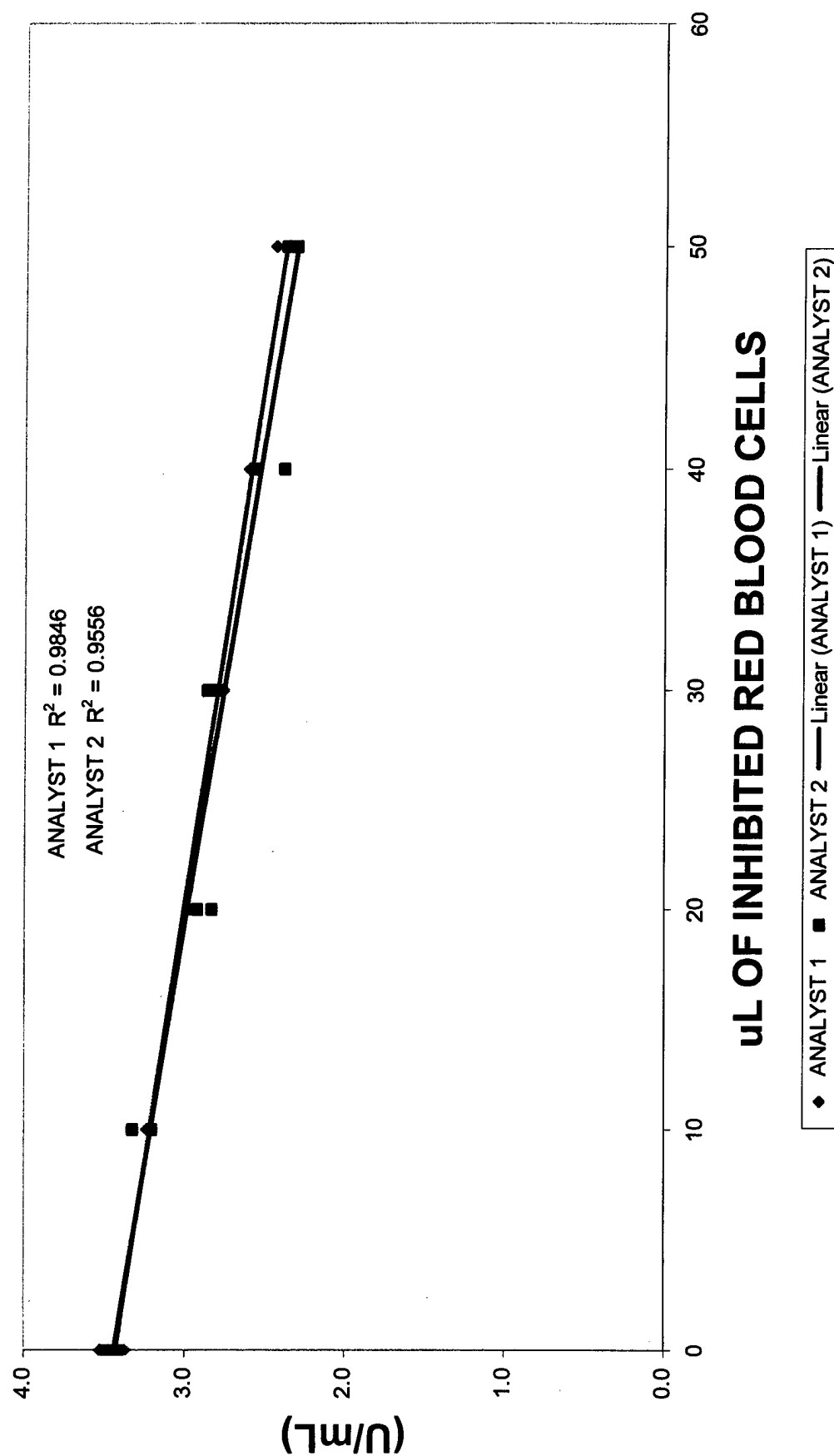
FIGURE 24.

PChE (387-48)
PARTIALLY INHIBITED BLOOD LINEARITY ANALYSIS

ANALYST 1 R^2 = 0.9532
ANALYST 2 R^2 = 0.8981
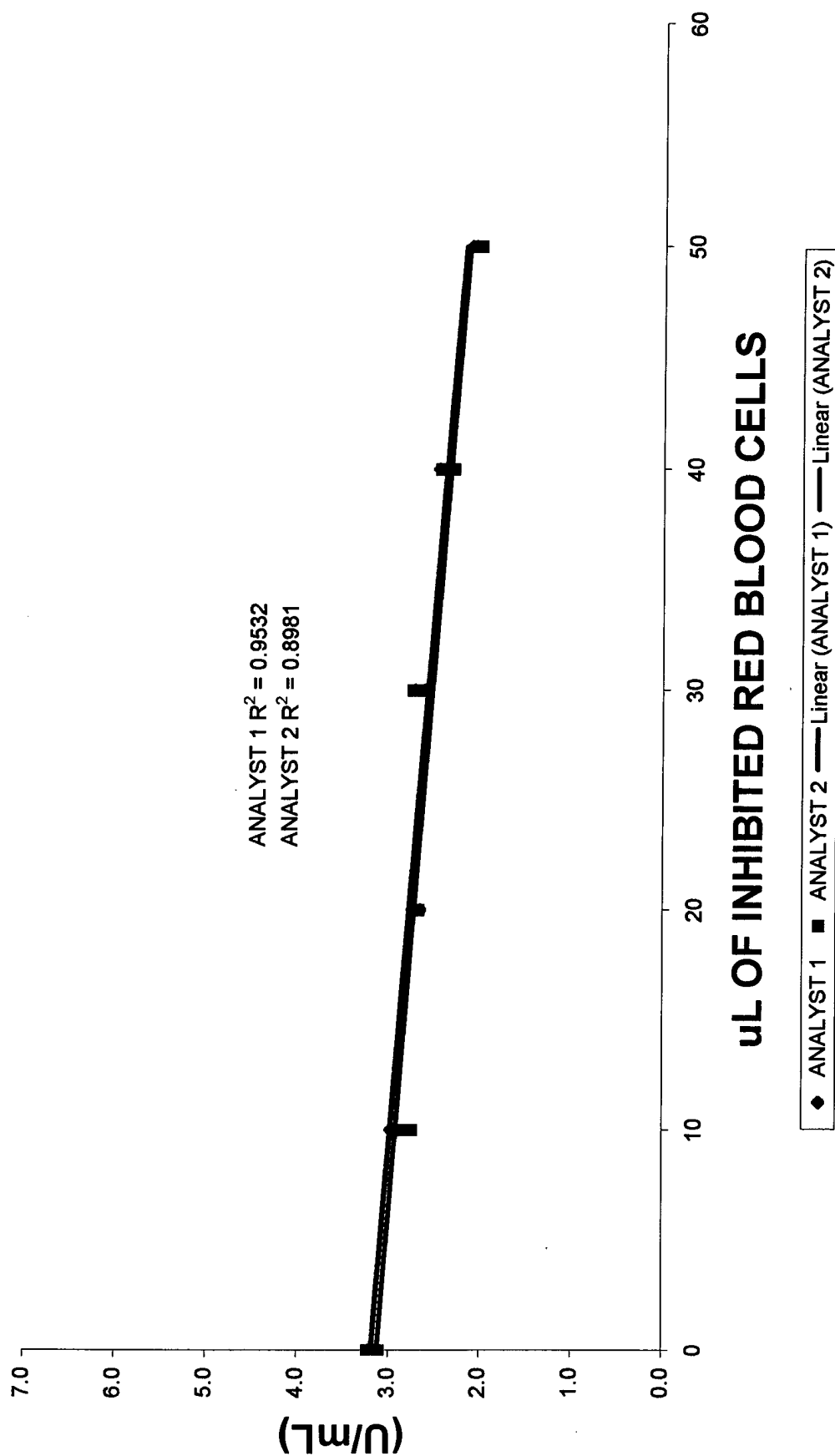
FIGURE 25.

COMPARISON OF INITIAL COBAS RESULTS (7/27/98) TO TEST MATE AChE 5-DAY AVERAGE RESULTS
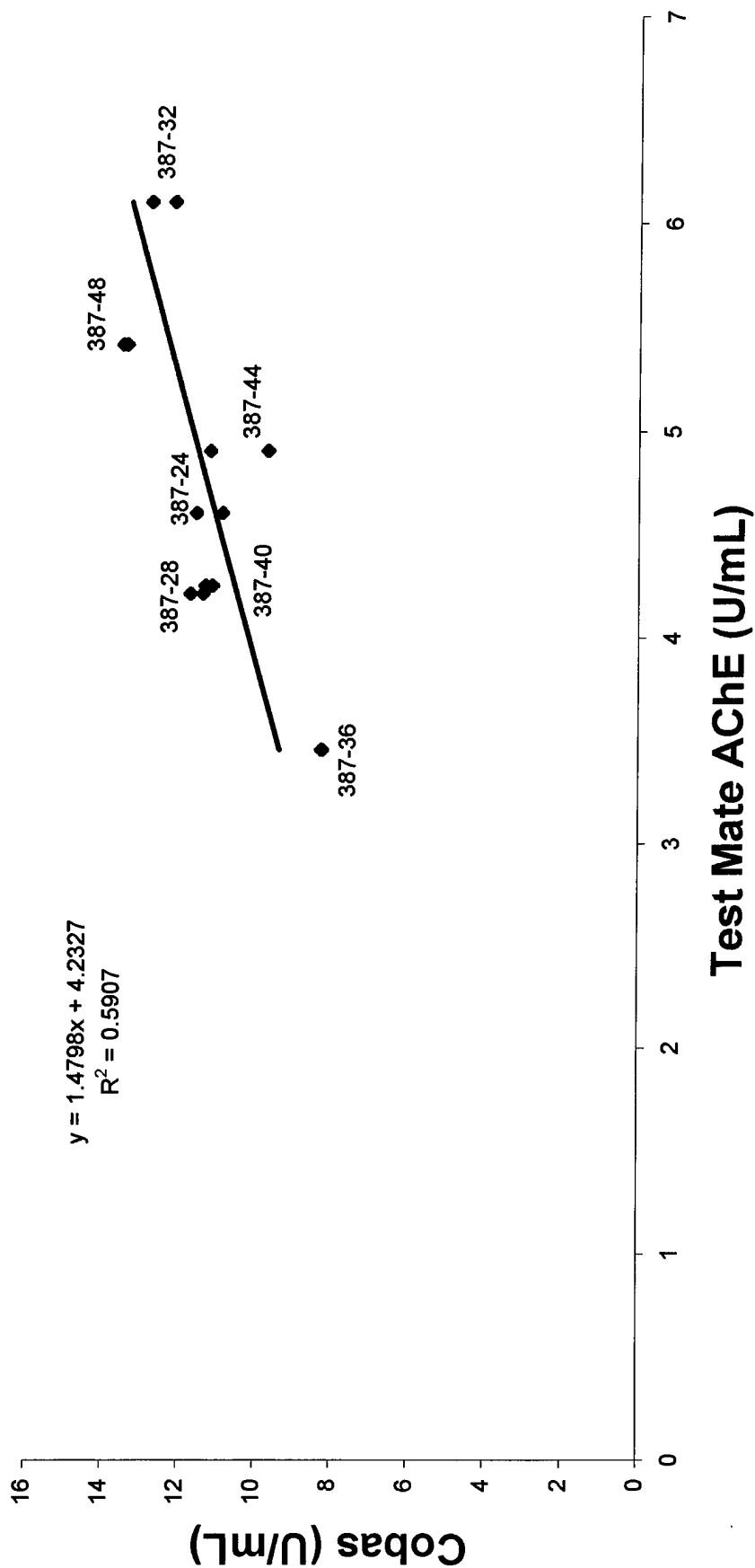
FIGURE 26.

COMPARISON OF COBAS RESULTS TO TEST MATE AChE RESULTS (8/19/98) DONOR 387-32

$y = 0.8926x + 0.3751$
$R^2 = 0.9942$
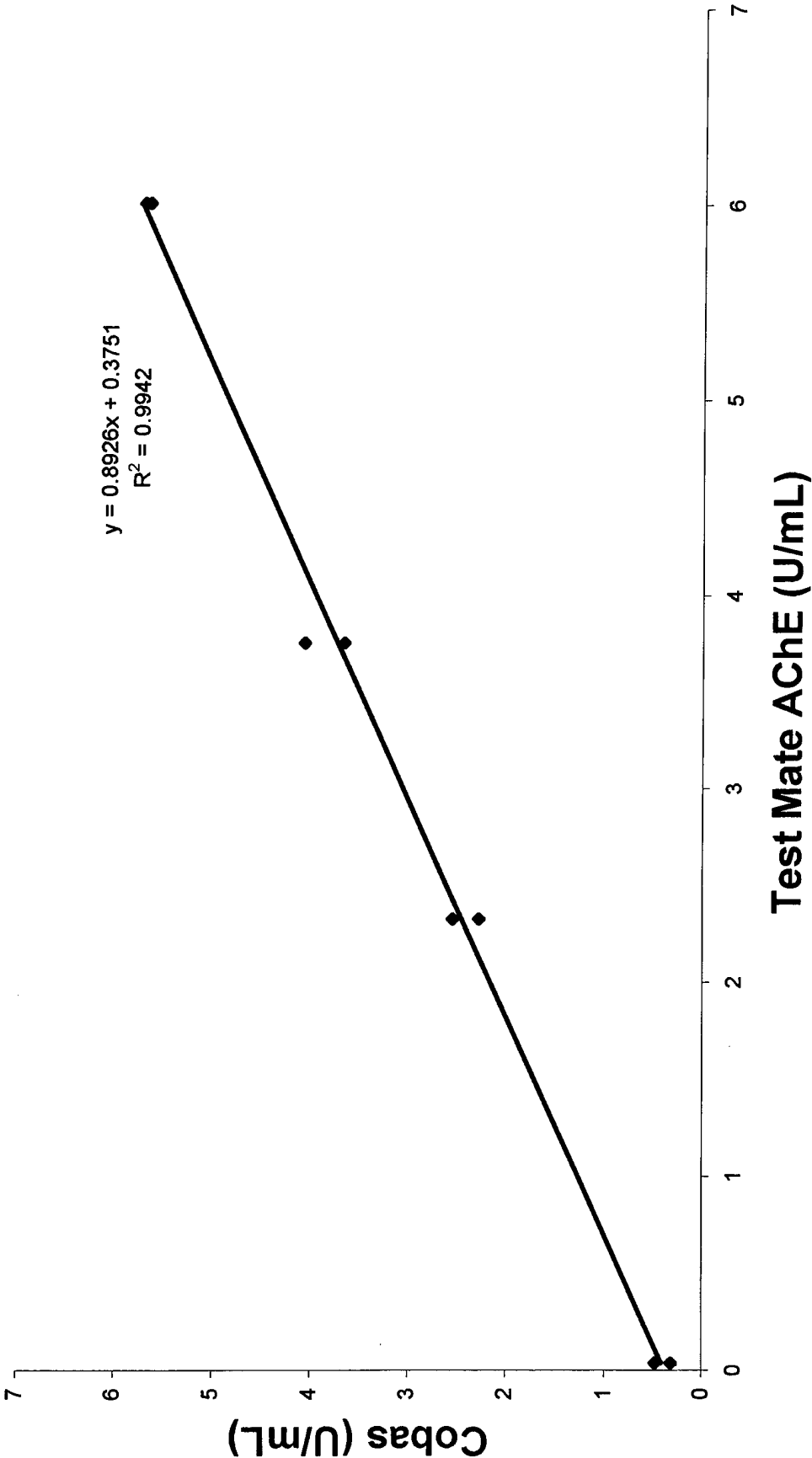
FIGURE 27.

COMPARISON OF COBAS RESULTS TO TEST MATE AChE
RESULTS (8/19/98) DONOR 387-36

$y = 1.1363x + 0.4359$
$R^2 = 0.9822$

Cobas (U/mL)
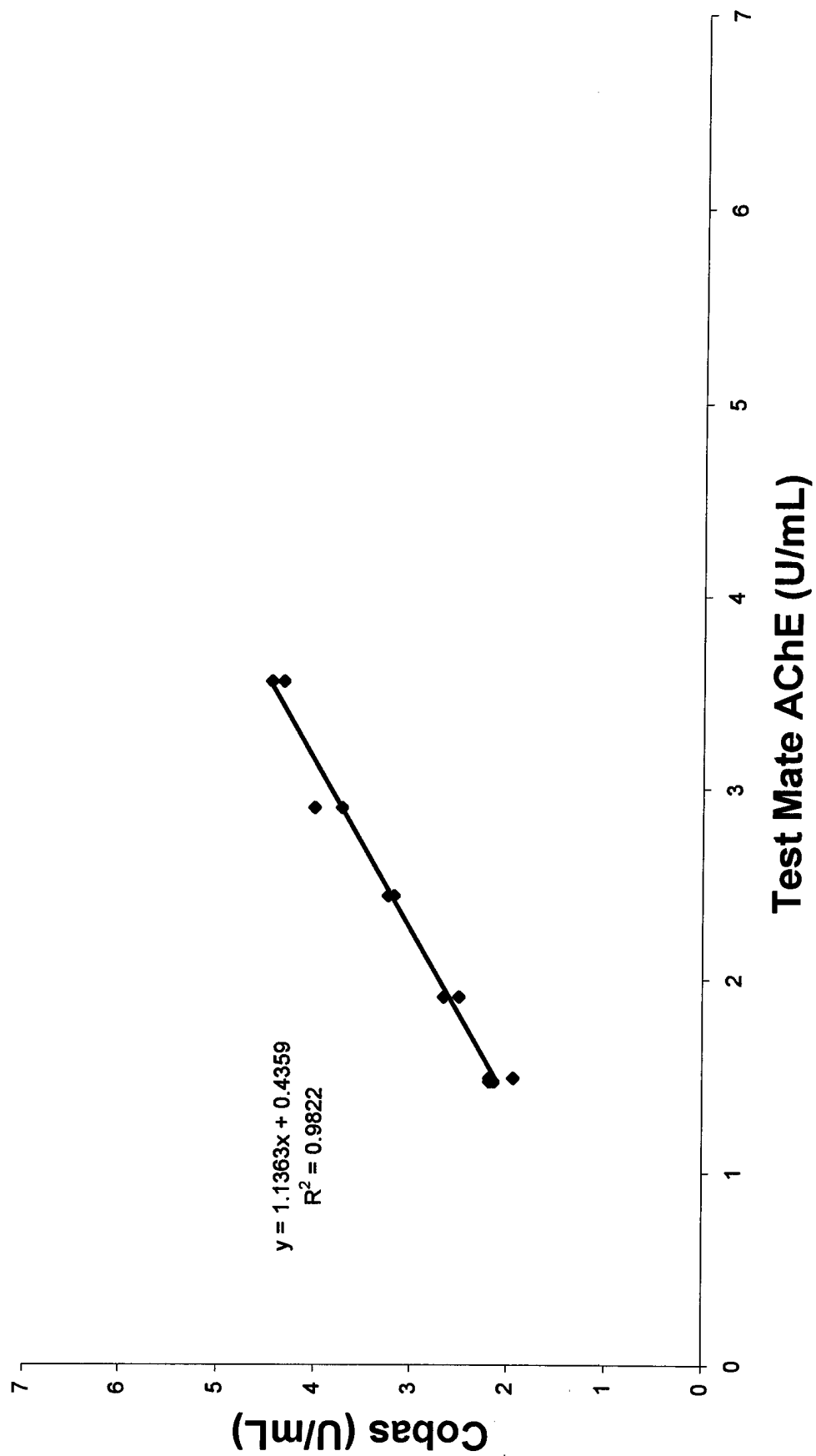
Test Mate AChE (U/mL)

FIGURE 28.

COMPARISON OF COBAS RESULTS TO TEST MATE AChE
RESULTS (8/19/98) DONOR 387-40

$y = 1.3926x - 0.187$
$R^2 = 0.9888$

Test Mate AChE (U/mL)

Cobas (U/mL)
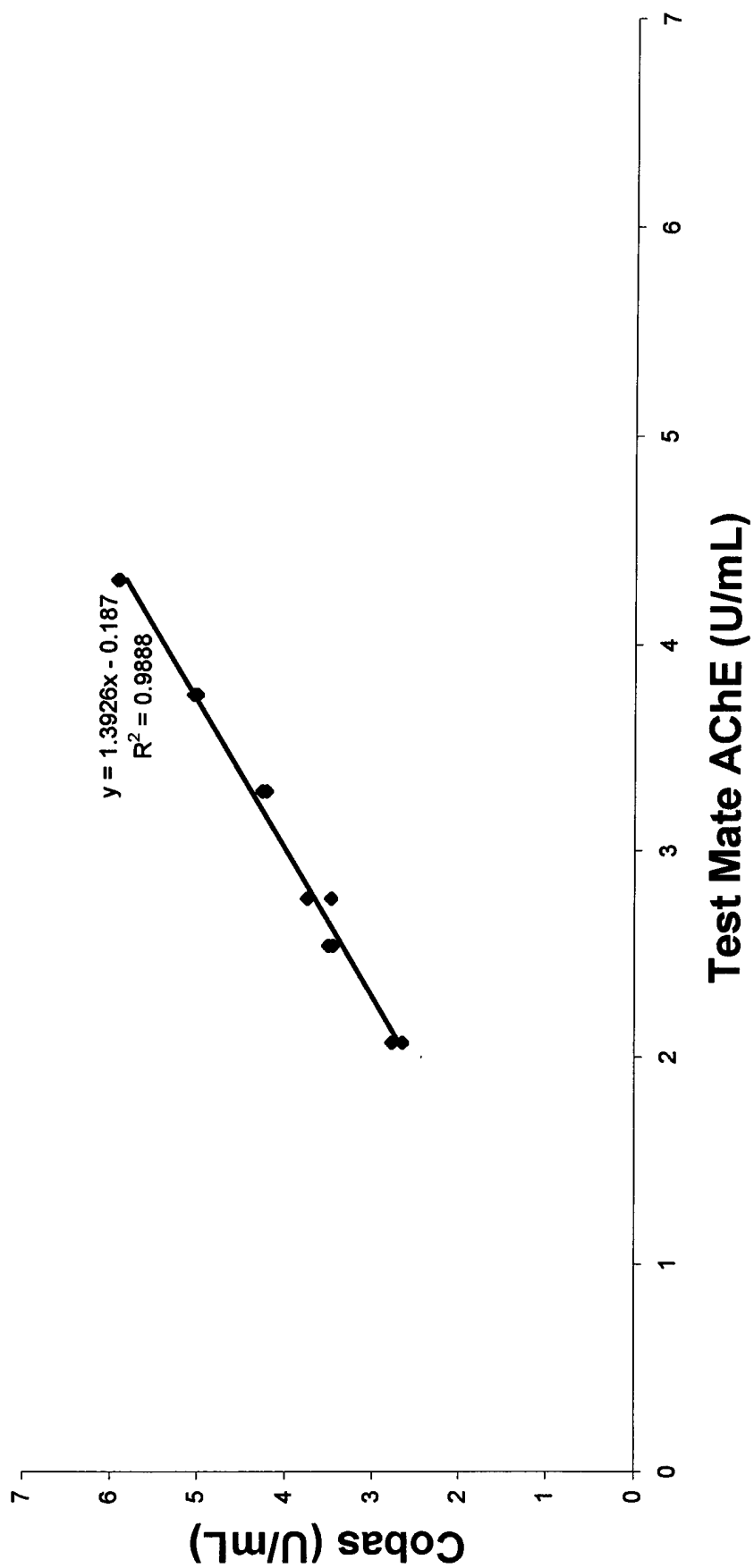
FIGURE 29.

COMPARISON OF COBAS RESULTS TO TEST MATE AChE RESULTS (8/19/98) DONOR 387-44

y = 1.3805x - 0.2606
R² = 0.9361

Test Mate AChE (U/mL)

Cobas (U/mL)
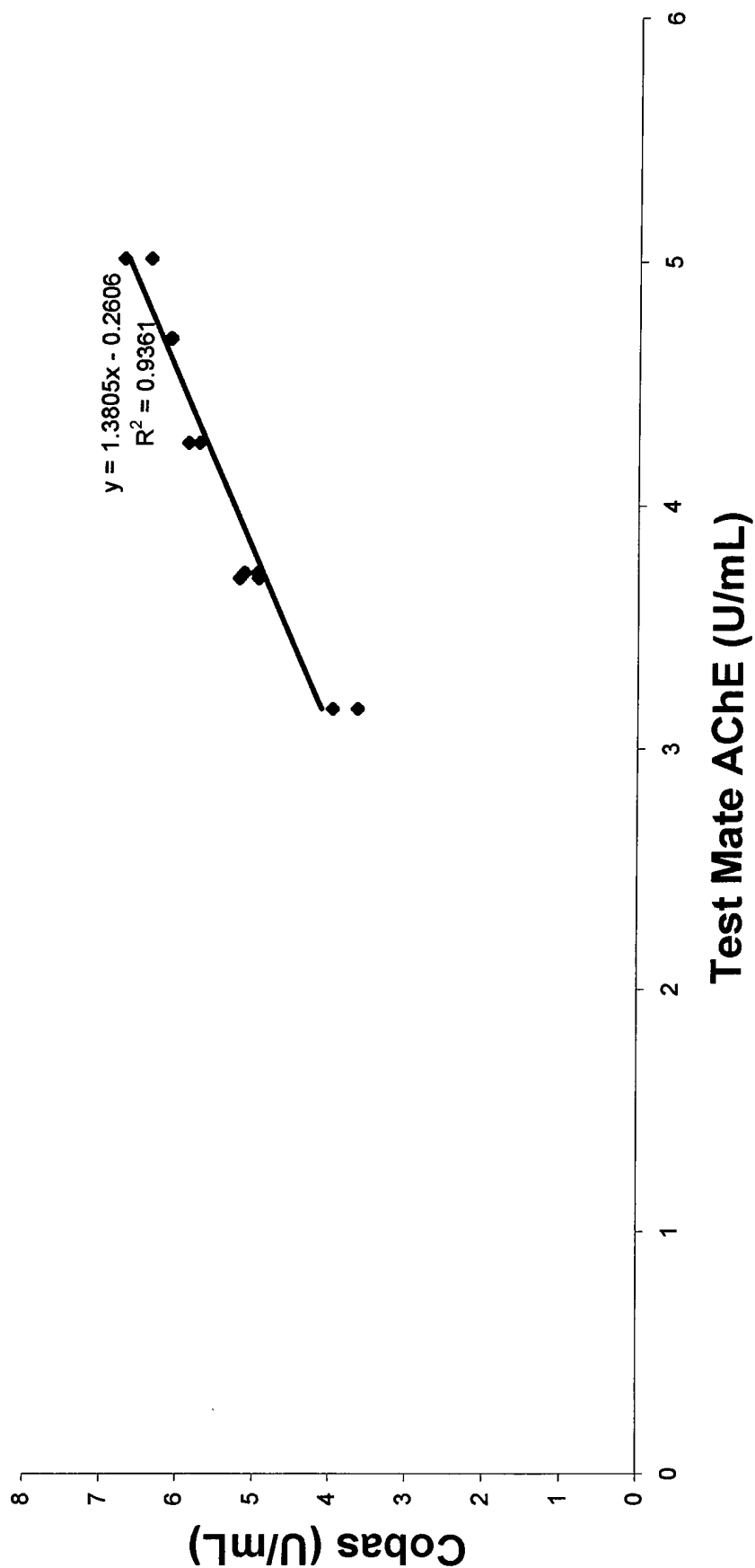
FIGURE 30.

COMPARISON OF COBAS RESULTS TO TEST MATE AChE RESULTS (8/19/98) DONOR 387-48
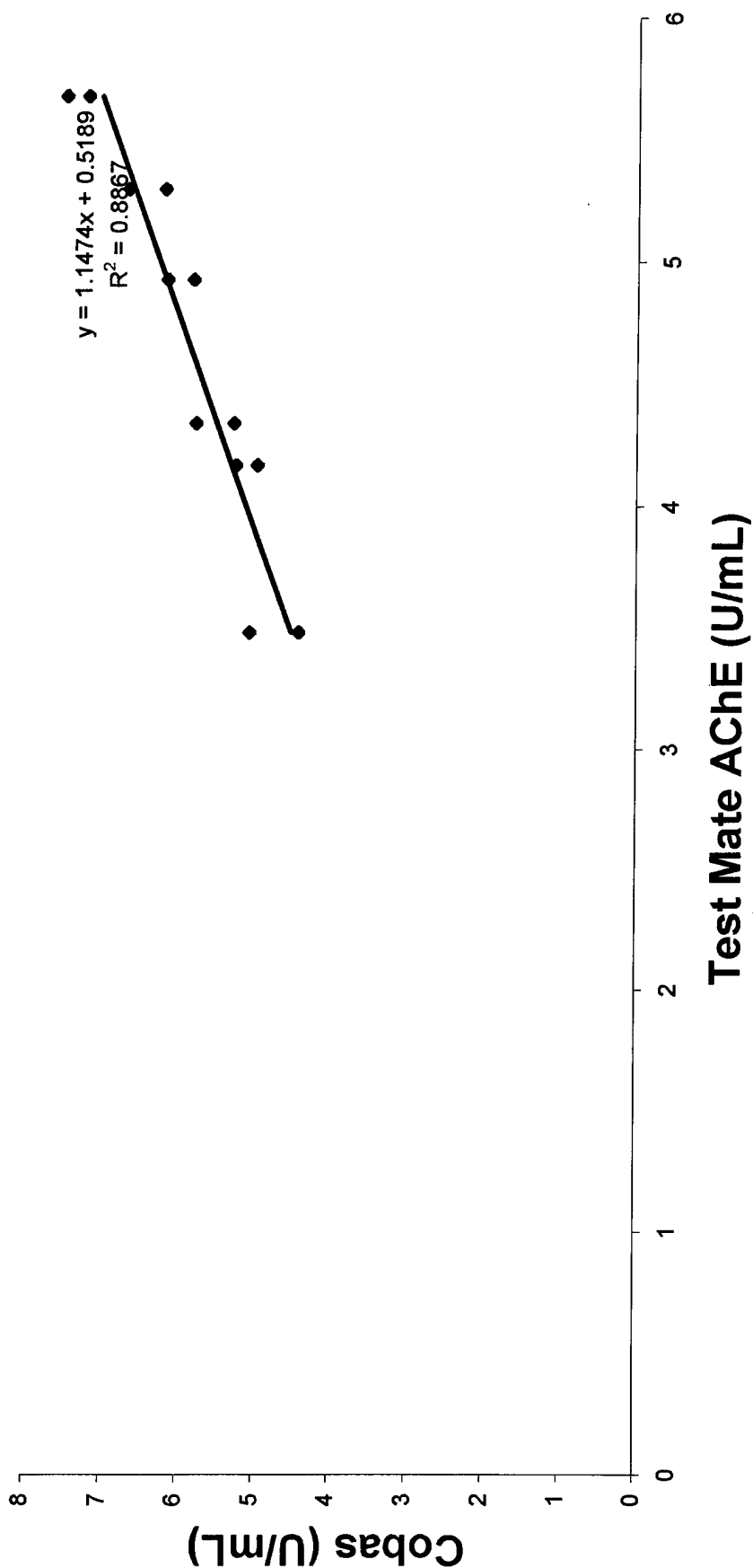
$y = 1.1474x + 0.5189$
$R^2 = 0.8867$

TASK 97-55
VALIDATION OF A GAS CHROMATOGRAPH/MASS SPECTROMETER METHOD FOR VERIFICATION OF NERVE AGENT EXPOSURE

1.0 INTRODUCTION

Battelle's Medical Research and Evaluation Facility (MREF) was requested by the United States Army Medical Research Institute of Chemical Defense (USAMRICD) to validate a gas chromatograph/mass spectrometer method for the detection of the metabolites of nerve agents, phosphonic acids, in human urine. The work was done under Contract DAMD 17-89-C-9050. The method was validated using the procedures reported in Technical Bulletin (TB MED 296), Chapter 4, "Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman, GF, and Cyanide", Headquarters, Department of the Army, May 1996.

It has been known at least since the mid-1960s that animals (including humans) exposed to a nerve agent metabolize the agent to the corresponding phosphonic acid and that the major route of elimination is through the urine.[1] Numerous studies have been done to develop analytical methods for phosphonic acids in environmental and other matrices less complex than that of biological systems.[1] Yet it is the biological matrix that is of increasing importance due to heightened world tension.

It is of critical importance to the military not only to protect their forces from weapons of mass destruction, but in the event of exposure to be able to identify and quantitate the agent, quickly and accurately, for treatment purposes. One of the main advantages of a chromatographic method is that the different phosphonic acids can be detected independently of one another, thus verifying not only nerve agent exposure but also which agent was involved.

The use of a combined gas chromatography/mass spectrometry (GC/MS) method, particularly one using Single Ion Monitoring (SIM), not only increases specificity but also lowers the detection limit due to increased dwell time at particular mass to charge ratios (m/e). When using internal standards that are deuterated isotopes of the analytes to be determined, a method using mass spectrometry is a necessity. Once an analytical method has been developed, it must be shown that it can be reproduced on a daily basis by analysts in other laboratories and with samples from more that just one source. To determine bias as a result of the matrix, urine

from different donors was analyzed. The calibration curves and responses of samples from different sources were compared.

2.0 EXPERIMENTAL PROCEDURES

A summary of the method is described in the following paragraphs with a detailed description of the experimental procedures provided in MREF Method No. 19-1/Chemistry located in Appendix F.

Isopropyl methylphosphonic acid (IMPA), pinacolyl methylphosphonic acid (PMPA) and cyclohexyl methylphosphonic acid (CMPA), the metabolites of GB, GD and GF, respectively, were the analytes assessed with this method. D_3-PMPA was used as the internal standard for PMPA and CMPA. D_3-IMPA was used as the internal standard for IMPA. With the exception of the PMPA, which was purchased from Aldrich Chemical Co., the analytes and internal standards were synthesized by Dr. Allison Fentiman of Battelle.

For the test urine matrices, approximately one liter of urine was collected from each of nine volunteers over a one to two day period for use in this study. After one liter of urine had been received from a particular donor, the urine was thoroughly mixed and 10-12 mL portions were aliquoted into labeled, plastic screw-cap centrifuge tubes. The centrifuge tubes were placed in test tube racks, which were enclosed in a plastic bag and stored in a –20 ± 10 C freezer. Sample aliquots were thawed only as needed for testing.

A 1 µg/mL mixed IMPA, PMPA and CMPA working standard and a 5 µg/mL mixed d_3-IMPA and d_3-PMPA working standard were prepared in water from the neat materials. A series of aqueous and urine standards were prepared for analysis by spiking nine microcentrifuge tubes containing 1 mL of each matrix with 0, 5, 10, 20, 40, 100, 125, 175 and 200 µL of the 1 µg/mL mixed IMPA, PMPA and CMPA working standard. To each of these nine microcentrifuge tubes was added 20 µL of the 5 µg/mL mixed d_3-IMPA and d_3-PMPA internal standard so that the concentration of the internal standards would be at the approximate mid-concentration range of the analytes, 100 ng/mL. By carrying out the analysis in various matrices, matrix related biases in the data should become evident.

The pH of the standards was adjusted to less than 1 to ensure that the phosphonic acids were un-ionized before the sample extraction step using C18 solid-phase extraction cartridges. The cartridges were conditioned with methanol followed by high purity water.

The prepared standards in each microcentrifuge tube were added directly to the correspondingly labeled C18 cartridges. The solutions were forced through the cartridge using air from a syringe attached to the cartridge until no additional liquid could be seen exiting from the bottom. Each cartridge was rinsed with a 20 percent NaCl /0.1 N HCl aqueous solution. Two additional 5-mL volumes of air were gently pushed through each cartridge to ensure that all aqueous solution had been removed. The final elution solvent, methanol, was passed through the cartridges into microcentrifuge tubes containing 20 ± 2 mg of dry, powdered potassium carbonate. The methanol extracts were evaporated to dryness in a 85 ± 5 C heating block under a gentle flow of nitrogen.

After these sample preparation steps, methylene chloride containing the catalyst 18-crown-6 ether was added to the residue in each microcentrifuge tube, followed by the addition of derivatizing agent, pentafluorobenzyl bromide (PFBBr). The capped tubes were heated in a reaction block at 50 ± 5 C for one hour with vortexing every 15 min.

After derivatization, each sample tube was centrifuged and the entire liquid layer was transferred to a centrifuge tube. The methylene chloride was evaporated at room temperature under a stream of nitrogen and the residue was reconstituted with carbon tetrachloride, which was back extracted with water to quench any remaining derivatizing reagents and remove water soluble interferences. The sample tubes were mixed by vortexing and re-centrifuged to separate the layers. The carbon tetrachloride layer was transferred to an autosampler vial for analysis by GC/MS.

A Micromass Quattro II mass spectrometer set for selected ion monitoring (SIM) in the electron ionizaation mode (70eV) was used to analyze all samples. The ions used for quantitation were 256 m/e for the analytes and 259 m/e for the deuterated internal standards. The 303 m/e and 306 m/e ions were monitored for identification of IMPA, PMPA, d_3-IMPA, and d_3-PMPA. For CMPA, 181 m/e and 277 m/e ions were used for positive identification.

Samples were chromatographed using a Fisions/Thermoquest 8000 Series II equipped with an AS 800 autosampler using the following conditions:

Column   DB-5MS, 25 m, with a 0.2-mm internal diameter and 0.33 μm film thickness

Column Temperatures
         45 C for 1 min
         45 C to 190 C at a rate of 20 C/min
         190 C for 5 min
         190 C to 320 C at a rate of 20 C/min
         320 C for 2 min

Typical retention times under these conditions are listed in Table 1.

Table 1. Typical Retention Times for Phosphonic Acids and Internal Standards
(± 0.05 min)

IMPA	10.6
PMPA	13.8, 14.2 (isomer 1, isomer 2)
CMPA	16.5
D_3-IMPA	10.5
D_3-IMPA	13.7, 14.1 (isomer 1, isomer 2)

The two isomers of PMPA and d_3-PMPA were integrated as separate compounds. Typical full scan spectra for the analytes and internal standards are shown in Figures 1-5. Examples of extracted ion chromatograms are shown in Figures 6-8.

3.0 VALIDATION

The urine of seven donors was arbitrarily chosen from the nine that were originally collected. A full set of nine concentration levels of the three phosphonic acids with internal standards were prepared in water and in the seven urine samples. Thus, for each set a total of 72 samples (9 concentration levels x 8 matrices) were analyzed using the method. The analysis process was repeated a total of five times on non-consecutive days for the validation data set. Individual calibration curves were generated for each of the eight individual matrices, and the concentrations of the analytes calculated using the regression lines which had been determined.

In addition, concentration levels of the analytes were re-calculated for all samples in each set using three different methods. The first method used the water standards for calibration, the second urine donor #2 standards for calibration, and the third standards in urine donor #4.

The differences between the calculated analyte concentrations and the spiked analyte concentrations were tabulated for all analyses. For this study, the definition of quantitation limit was the concentration at which the average percent difference and the percent RSD of the difference between spiked and actual analyte concentrations for the five day testing period was no more than 15 percent.

The definition of detection limit was the concentration at which the analyte signal to noise ratio is approximately 3:1. The signal to noise ratio of the lowest 5 ng/mL standard was used to approximate this concentration, and samples at the calculated level were prepared and analyzed for verification.

4.0 RESULTS AND DISCUSSION

The forcing of additional air through the C18 cartridges and the drying of the potassium carbonate were found to be critical to the success of the method. Residual water in the methanol increases the solvent evaporation time and interferes in the subsequent derivatization. It is important that the potassium carbonate be dried by heating to 130 ± 10 C overnight before use since it is used to dry the methanol and the potassium acts as a catalyst in the derivatization.[2]

Linearity

Examples of regression lines for each analyte in both water and urine matrices are shown in Figures 9-16. As listed in the text for each figure, the correlation coefficients are all greater than 0.99. The average values of the correlation coefficient over the five trials are listed in Table 2, with percent RSD values for each set in parentheses. The water values are an average of the one water sample over five trials. Urine values are an average of 35 values (seven urine samples over five trials).

Table 2 – Correlation Coefficient Summary

	IMPA	PMPA-Isomer 1	PMPA-Isomer 2	CMPA
Water	0.999 (0.0)	0.996 (0.4)	0.996 (0.6)	0.997 (0.2)
Urine	0.999 (0.2)	0.998 (0.5)	0.997 (0.8)	0.997 (0.3)

A correlation coefficient of 0.99 or greater for regression lines is considered acceptable for most applications. This value also should be obtainable in analyses on different days. From the values listed in Table 2, a linear regression model fits the data in the range of 5-200 ng/mL IMPA, PMPA and CMPA. A percent RSD of less than 0.8 indicates the expected reproducibility of linearity over time.

Quantitation Limit

Tables 3-10 contain the calculated analyte concentrations in water and each of the seven urine samples using the regression line generated for its own matrix. The average of the 5 trials, the percent difference between spiked and calculated concentrations, standard deviation and percent RSD for the spiked and calculated differences have also been included. Table 11 is a summary of the percent RSD values presented in Tables 3-10. Below a concentration of 20 ng/mL, the difference between the spiked and calculated analyte concentration approaches 15 percent, particularly in the samples with a water matrix. For this reason, a quantitation limit of 20 ng/mL was established.

Matrix Interferences

Since it may not be possible to obtain pre-exposure urine of victims for standards preparation, Battelle evaluated the use of water and different urine samples as calibration matrices. For this reason, the analyte concentrations in all samples of each set were recalculated using either water, urine donor #2 or urine donor #4 samples for the calibration standards. The results are summarized in Table 12. From these data, it can be seen that in most situations pooled urine will give a better correlation between spiked and calculated levels except in the case of IMPA, where the aqueous standards are more reproducible. The method of standard additions, overspiking of post-exposure urine with the analyte of interest, should be considered if increased accuracy is required.

Detection Limit

Using the Masslynx software on the mass spectrometer, the signal to noise ratios for the 5 ng/mL standard of each analyte were calculated and the results are summarized in Table 12. With a signal to noise ratio of 3:1 as the criteria for the detection limit, an analyte concentration of 0.3-0.5 ng/mL was calculated. As can be seen from the data summary in Table 13, a concentration of 0.3 ng/mL closely approximates a 3:1 signal to noise ratio.

5.0 CONCLUSION

This method is an acceptable means of measuring IMPA, PMPA and CMPA concentrations in human urine in the range of 5-200 ng/mL. All three phosphonic acids can be detected at levels of 0.3 ng/mL and quantitated at 20 ng/mL. Although the calibration curves are linear down to 5 ng/mL, the increased standard deviation at the low levels may preclude accurate measurements at these concentrations.

The pre-exposure urine of the patient is the preferable matrix for generating a calibration curve; however, when this is not available, pooled urine or water may be used. Over-spiking of post-exposure urine should be considered if improved accuracy is required.

Since the derivatization is a critical step in the analysis, care must be taken to ensure that all water is removed from the C18 cartridge before eluting the analytes with methanol. It is important that the potassium carbonate be dried by heating to 130 ± 10 C overnight before use.

6.0 REFERENCES

1. Technical Bulletin (TB MED 296), "Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman, GF, and Cyanide", Headquarters, Department of the Army, May 1996.

2. Pentafluorobenzyl Bromide, Product Specification Bulletin, T497103, Supelco, 1997.
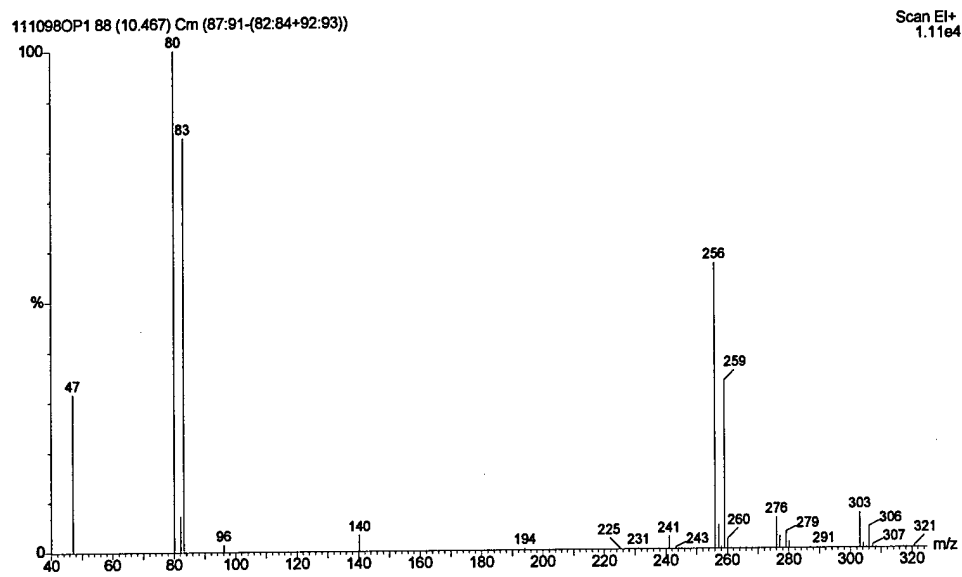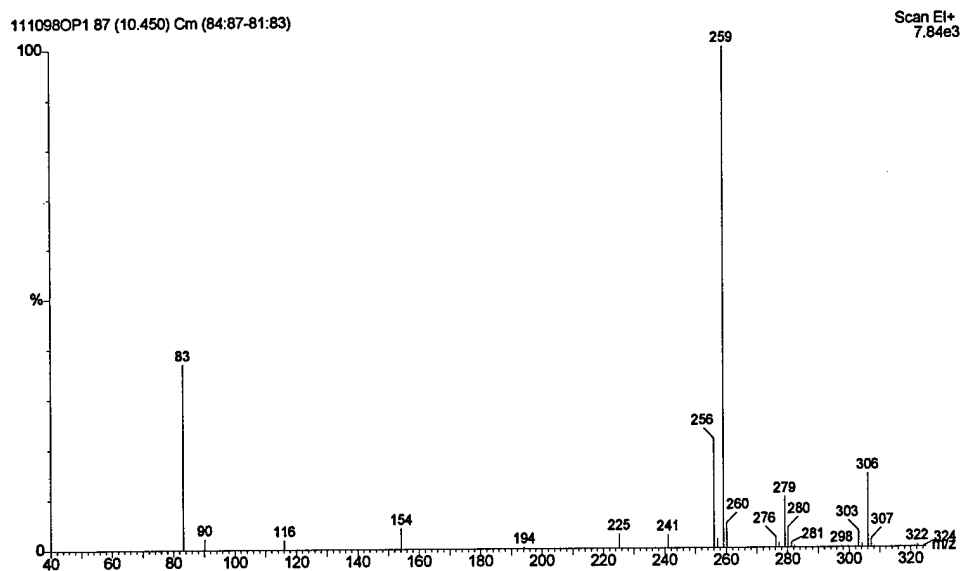
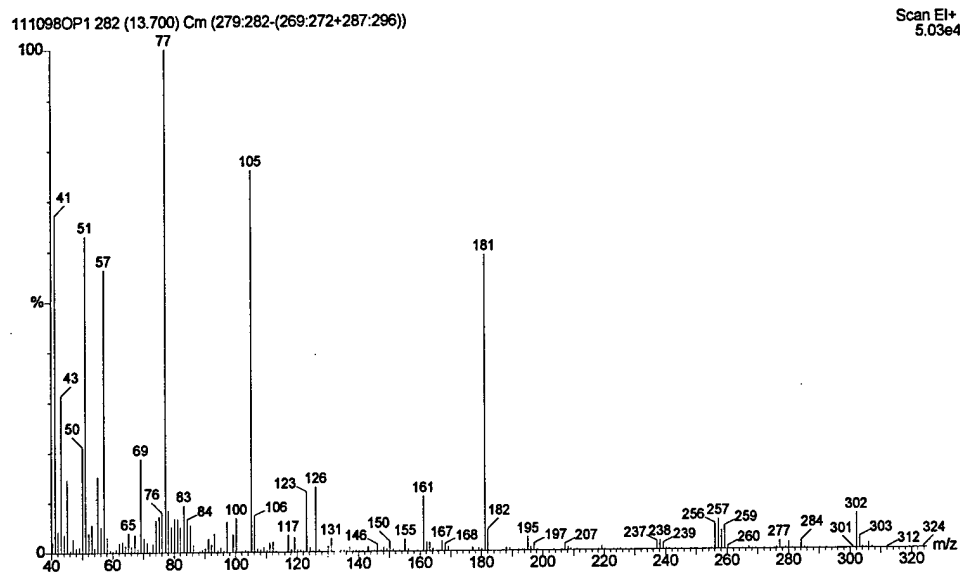Figure 1. Spectrum of PFB-IMPA

Figure 2. Spectrum of PFB-d₃-IMPA

111098OP1 282 (13.700) Cm (279:282-(269:272+287:296))

Scan EI+
5.03e4

Figure 3. Spectrum of PFB-PMPA

111098OP1 279 (13.650) Cm (276:279-(269:272+284:290))

Scan EI+
1.70e4
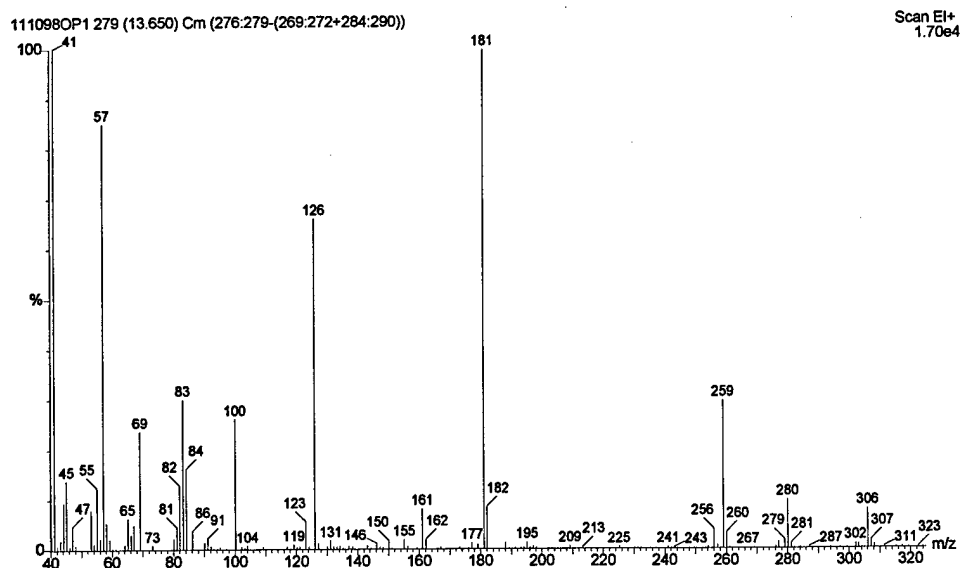
Figure 4. Spectrum of PFB-d₃-PMPA

111098OP1 449 (16.484) Cm (447:449-(433:441+454:458))   Scan EI+
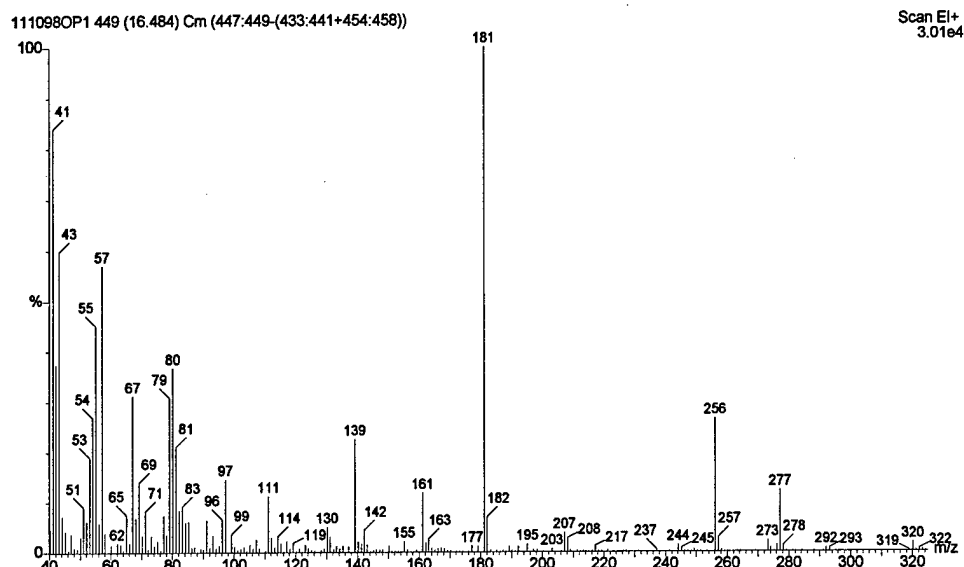   3.01e4
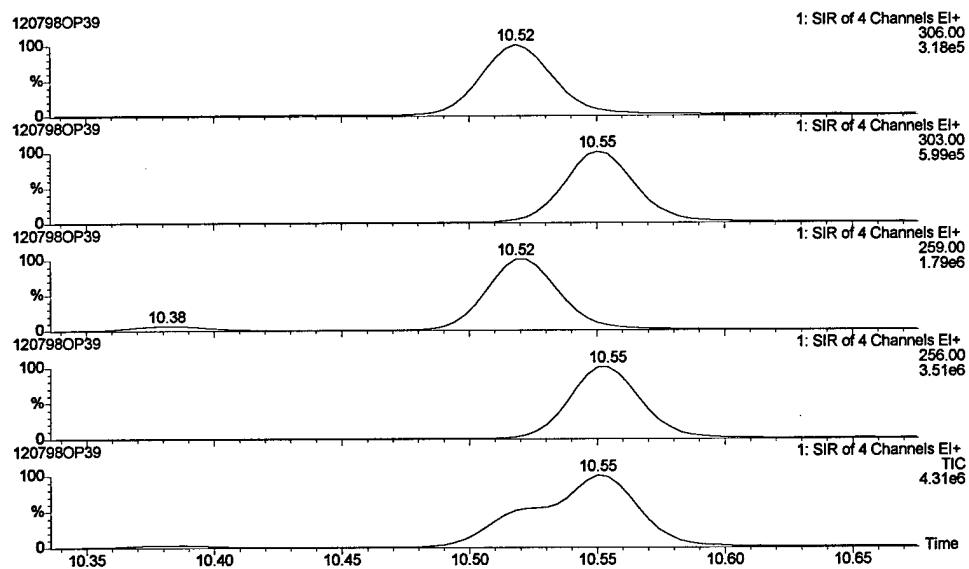

Figure 5. Spectrum of PFB-CMPA
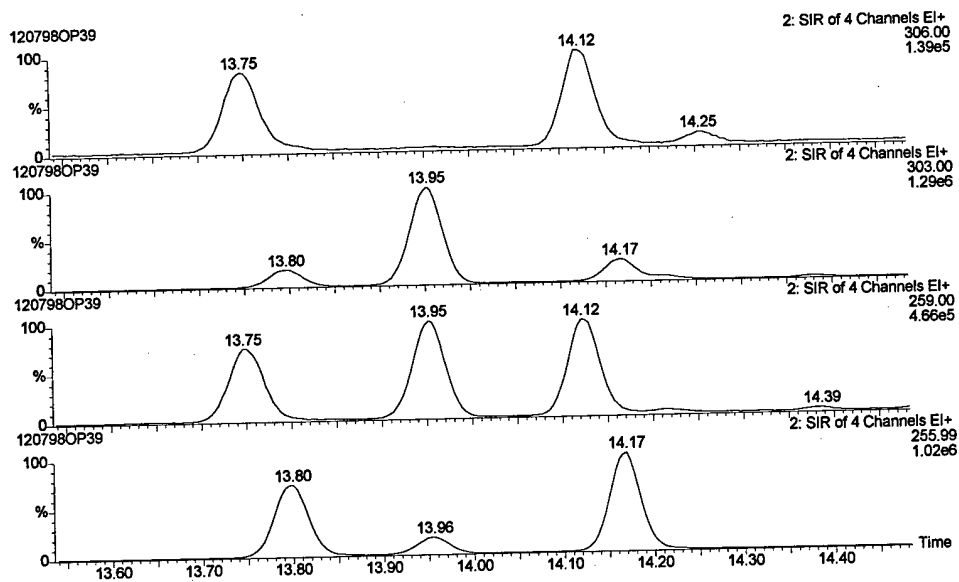

Figure 6. SIM for PFB-IMPA and PFB-d₃-IMPA
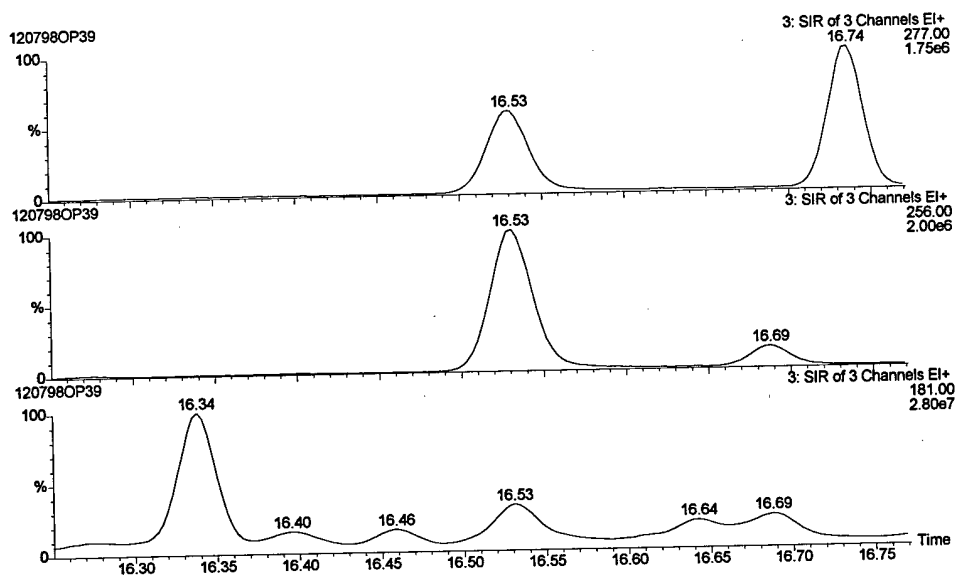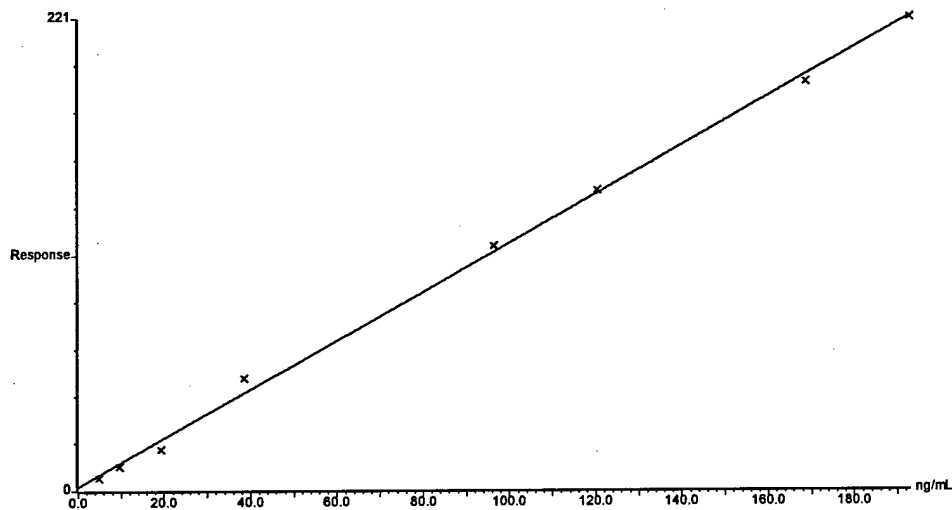
Figure 7. SIM for PFB-PMPA and PFB-d₃-PMPA

Figure 8. SIM for PFB-CMPA

Compound 1 name: PFBBR Der. of IMPA
Correlation coefficient: r = 0.999077, r^2 = 0.998155
Calibration curve: 1.13539 * x + 1.72897
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None

Figure 9. Regression Line for IMPA in Water

Compound 5 name: PFBBR Der. PMPA Isomer #1
Correlation coefficient: r = 0.999588, r^2 = 0.999176
Calibration curve: 1.25426 * x + 1.27740
Response type: Internal Std (Ref 3), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None
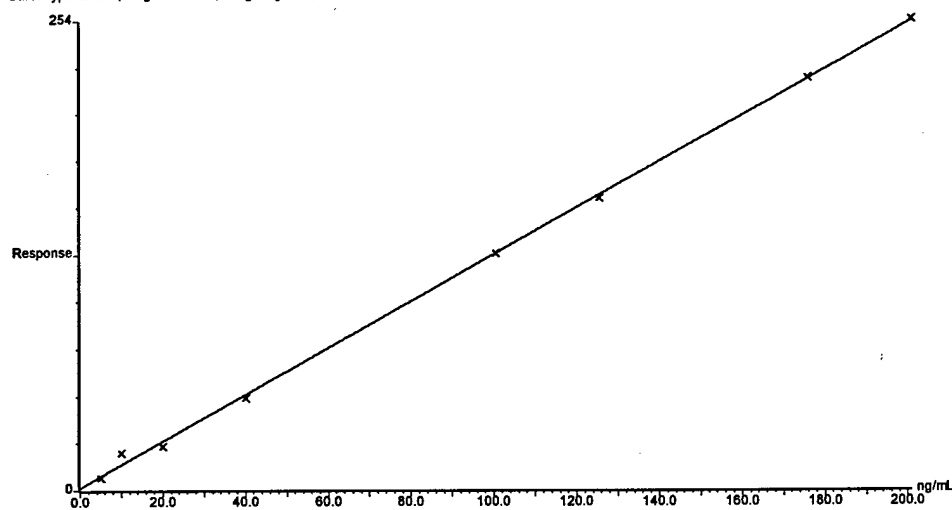
Figure 10. Regression Line for PMPA-Isomer 1 in Water

Compound 6 name: PFBBR Der. PMPA Isomer #2
Correlation coefficient: r = 0.999438, r^2 = 0.998876
Calibration curve: 1.31836 * x + 1.27308
Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None

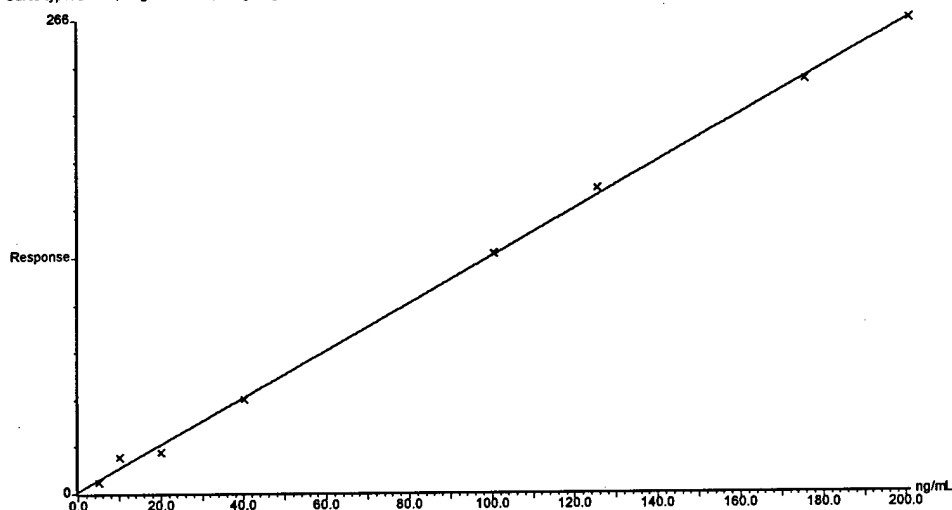**Figure 11. Regression Line for PMPA-Isomer 2 in Water**

Compound 7 name: PFBBR Der. CMPA
Correlation coefficient: r = 0.998761, r^2 = 0.997523
Calibration curve: 1.68482 * x + -2.48245
Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None

Figure 12. Regression Line for CMPA in Water

Compound 1 name: PFBBR Der. of IMPA
Correlation coefficient: r = 0.998690, r^2 = 0.997382
Calibration curve: 1.14773 * x + -1.15106
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None
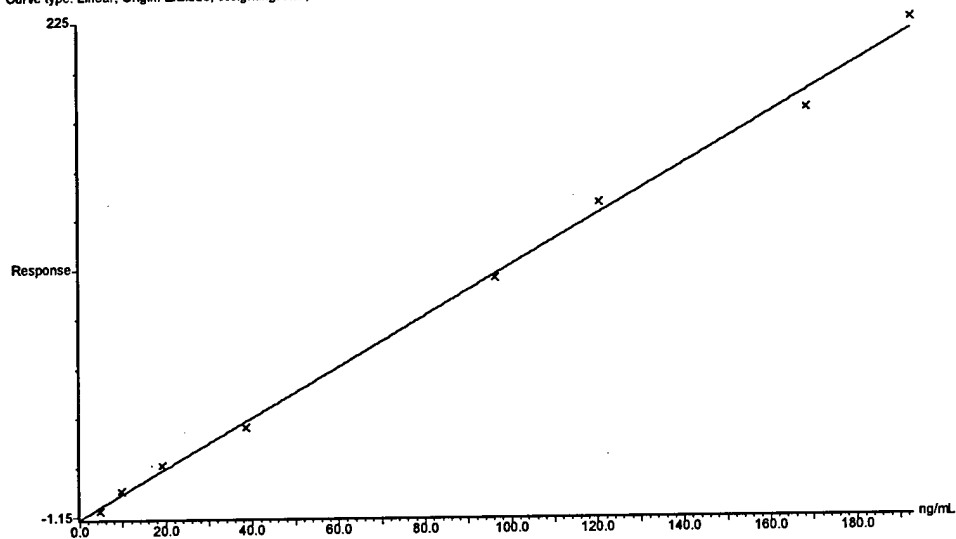
Figure 13. Regression Line for IMPA in Urine Donor #1

Compound 5 name: PFBBR Der. PMPA Isomer #1
Correlation coefficient: r = 0.999623, r^2 = 0.999246
Calibration curve: 1.24538 * x + -0.913253
Response type: Internal Std (Ref 3), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None
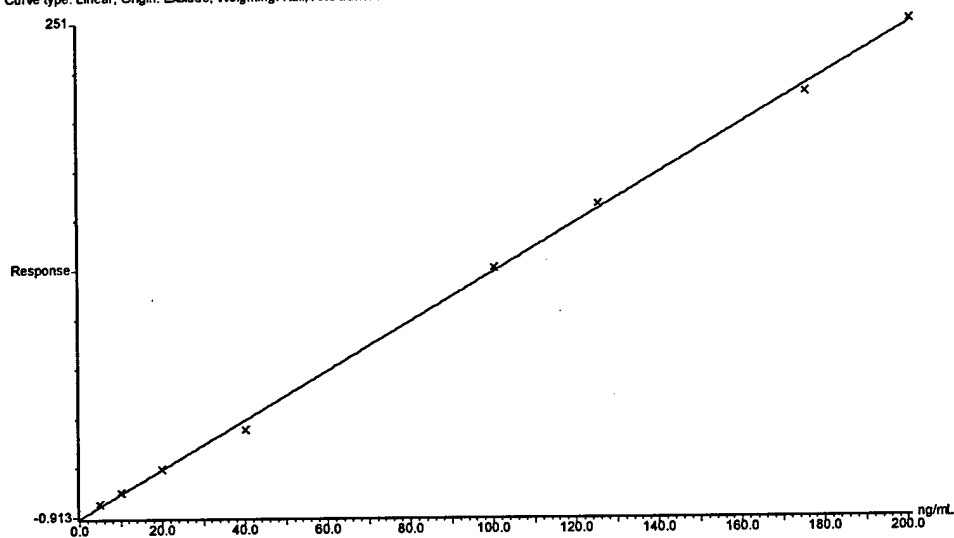
Figure 14. Regression Line for PMPA-Isomer 1 in Urine Donor #1

Compound 6 name: PFBBR Der. PMPA Isomer #2
Correlation coefficient: r = 0.999122, r^2 = 0.998245
Calibration curve: 1.25588 * x + -0.879235
Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None

Figure 15. Regression Line for PMPA-Isomer 2 in Urine Donor #1

Compound 7 name: PFBBR Der. CMPA
Correlation coefficient: r = 0.996589, r^2 = 0.993189
Calibration curve: 1.51529 * x + 0.396225
Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
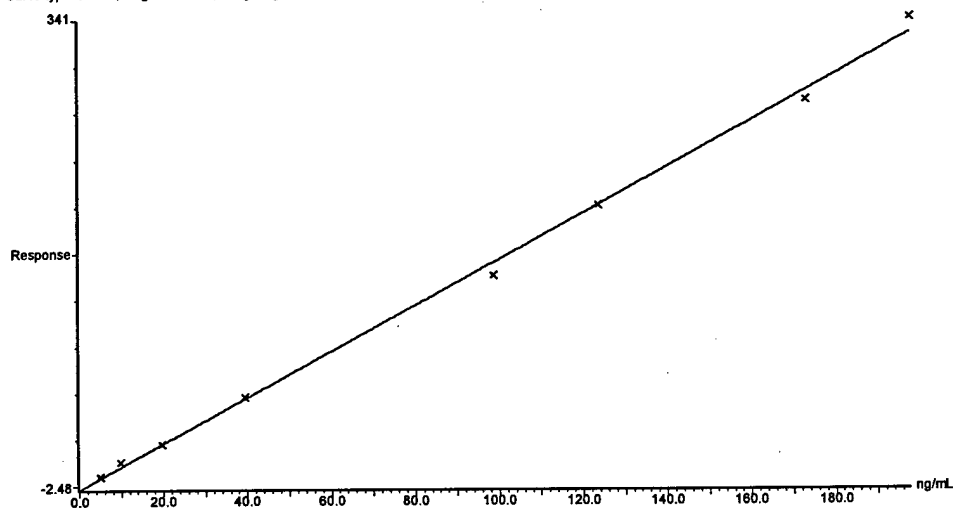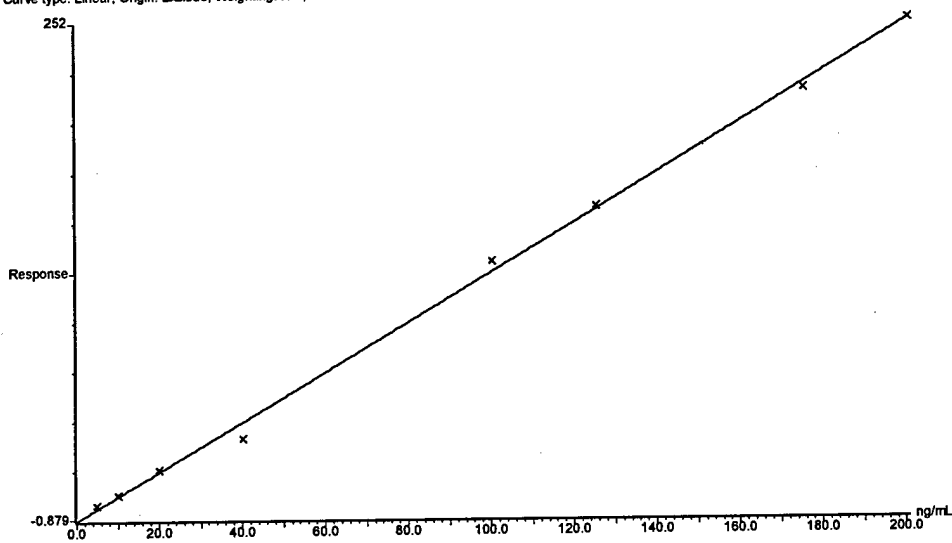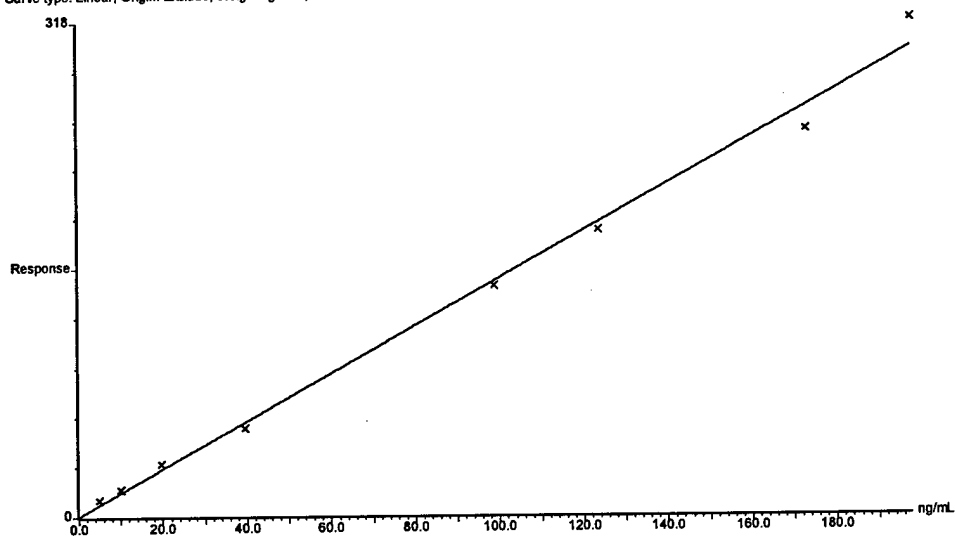Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None

Figure 16. Regression Line for CMPA in Urine Donor #1

Table 3. Calculated Concentrations of Phosphonic Acids in Water

Spiked Concention Levels of Phosphonic Acids in Water (ng/mL)	Calculated Concentrations ng/mL					Average	Percent Difference Between Calculated and Spiked	STDEV	% RSD
	Analyis 1 12/4/98	Analysis 2 12/9/98	Analysis 3 12/14/98	Analysisa 4 12/17/98	Analysis 5 12/22/98				
IMPA									
4.82	6.3	4.7	3.6	2.9	9.7	5.44	11.4%	2.7	49.7%
9.64	8.7	10.2	8.4	8.3	8.8	8.88	8.6%	0.8	8.6%
19.3	17.9	18.8	15.1	17.1	21.1	18.00	7.2%	2.2	12.3%
38.6	34.9	36.4	44.4	48.0	31.9	39.12	1.3%	6.8	17.3%
96.4	103.9	99.2	99.0	93.3	95.7	98.22	1.9%	4.0	4.1%
120.5	120.6	121.8	121.8	118.6	120.0	120.56	0.0%	1.3	1.1%
168.7	167.0	167.3	165.9	167.1	170.6	167.58	0.7%	1.8	1.1%
192.8	191.3	192.2	192.5	195.4	192.9	192.86	0.0%	1.5	0.8%
PMPA ISOMER 1									
5.02	21.1	1.9	4.2	5.9	7.7	8.16	38.5%	7.5	92.4%
10.04	3.6	6.8	14.8	10.7	9.2	9.02	11.3%	4.2	46.6%
20.08	14.4	15.3	17.7	18.9	21.7	17.60	14.1%	2.9	16.6%
40.16	31.5	34.0	38.6	37.9	36.7	35.74	12.4%	2.9	8.2%
100.4	106.3	123.2	100.6	97.5	91.8	103.88	3.4%	12.0	11.6%
125.5	123.9	138.8	124.2	133.6	136.6	131.42	4.5%	7.0	5.3%
175.7	177.1	166.7	176.2	174.4	172.6	173.40	1.3%	4.1	2.4%
200.8	199.9	190.9	201.5	198.8	201.4	198.50	1.2%	4.4	2.2%
PMPA ISOMER 2									
5.02	21.9	1.2	3.8	4.8	5.6	7.46	32.7%	8.2	110.5%
10.04	2.2	6.4	14.4	9.8	8.5	8.26	21.5%	4.5	54.2%
20.08	14.5	15.0	16.4	18.4	21.9	17.24	16.5%	3.0	17.5%
40.16	32.0	34.0	39.1	39.5	37.0	36.32	10.6%	3.3	9.0%
100.4	103.4	126.5	101.1	98.5	100.7	106.04	5.3%	11.6	10.9%
125.5	127.4	138.6	128.5	134.6	131.6	132.14	5.0%	4.6	3.5%
175.7	177.6	165.7	174.2	176.0	170.3	172.76	1.7%	4.8	2.8%
200.8	198.6	190.3	200.3	196.1	202.1	197.48	1.7%	4.6	2.3%
CMPA									
4.94	11.1	8.5	5.8	7.1	10.3	8.56	42.3%	2.2	25.6%
9.88	11.7	11.9	11.9	11.9	14.5	12.38	20.2%	1.2	9.6%
19.76	20.4	18.4	19.9	18.5	22.7	19.98	1.1%	1.8	8.8%
39.52	33.9	39.4	40.4	38.8	32.2	36.94	7.0%	3.6	9.9%
98.8	93.8	103.1	92.8	94.0	93.5	95.44	3.5%	4.3	4.5%
123.5	120.5	105.2	123.3	123.4	112.2	116.92	5.6%	8.0	6.8%
172.9	171.6	178.0	168.7	174.3	177.3	173.98	0.6%	3.9	2.2%
197.6	203.9	202.4	204.0	199.0	204.3	202.72	2.5%	2.2	1.1%

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Table 4. Calculanted Concentrations of Phosphonic Acids in Urine Donor I

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Spiked Concention Levels of Phosphonic Acids in Donor 1 Urine	Analysis 1 12/4/98	Analysis 2 12/9/98	Analysis 3 12/14/98	Analysisa 4 12/17/98	Analysis 5 12/22/98	Average	Percent Difference Between Calculated and Spiked	STDEV	% RSD
IMPA									
4.82	3.2	3.4	6.7	7.7	3.7	4.94	2.4%	2.1	42.5%
9.64	7.6	11.1	11.8	10.1	8.7	9.86	2.2%	1.7	17.4%
19.3	15.7	21.3	17.0	18.2	18.8	18.20	6.0%	2.1	11.5%
38.6	43.1	36.3	38.1	35.9	39.9	38.66	0.2%	2.9	7.6%
96.4	99.9	94.7	95.5	95.2	99.0	96.86	0.5%	2.4	2.5%
120.5	125.1	124.8	117.5	120.9	121.5	121.96	1.2%	3.1	2.6%
168.7	165.5	161.8	169.7	170.9	166.3	166.84	1.1%	3.6	2.2%
192.8	190.5	197.2	194.4	191.7	192.8	193.32	0.3%	2.6	1.3%
PMPA ISOMER 1									
5.02	4.5	6.1	7.0	6.5	4.7	5.76	12.8%	1.1	19.2%
10.04	9.0	10.5	12.0	10.7	10.2	10.48	4.2%	1.1	10.3%
20.08	20.1	20.4	17.8	20.1	21.7	20.02	0.3%	1.4	7.0%
40.16	38.2	36.5	41.0	38.7	38.6	38.60	4.0%	1.6	4.2%
100.4	103.6	101.8	96.9	97.6	99.9	99.96	0.4%	2.8	2.8%
125.5	130.1	127.5	123.8	125.8	126.3	126.70	0.9%	2.3	1.8%
175.7	174.1	172.7	176.0	178.3	174.5	175.12	0.3%	2.1	1.2%
200.8	198.2	202.1	203.3	200.0	201.7	201.06	0.1%	2.0	1.0%
PMPA ISOMER 2									
5.02	3.8	6.6	6.3	5.7	4.0	5.28	4.9%	1.3	24.7%
10.04	8.9	10.8	11.0	9.8	9.1	9.92	1.2%	1.0	9.7%
20.08	19.7	20.8	16.8	19.4	22.0	19.74	1.7%	1.9	9.8%
40.16	40.1	33.5	41.7	38.9	39.2	38.68	3.8%	3.1	8.0%
100.4	103.2	104.7	98.1	99.5	102.1	101.52	1.1%	2.7	2.7%
125.5	131.1	127.0	127.2	128.8	126.0	128.02	2.0%	2.0	1.6%
175.7	168.8	173.3	176.6	178.9	175.6	174.64	0.6%	3.8	2.2%
200.8	202.1	201.0	200.0	196.7	199.9	199.94	0.4%	2.0	1.0%
CMPA									
4.94	6.2	6.8	7.4	5.1	5.5	6.20	20.3%	0.9	15.1%
9.88	11.0	11.4	12.7	8.3	10.0	10.68	7.5%	1.6	15.4%
19.76	19.1	22.2	19.4	22.2	17.9	20.16	2.0%	1.9	9.6%
39.52	36.1	37.2	37.6	43.7	36.7	38.26	3.3%	3.1	8.1%
98.8	100.3	96.6	91.6	95.7	105.3	97.90	0.9%	5.2	5.3%
123.5	126.4	120.2	124.3	116.7	123.8	122.28	1.0%	3.8	3.1%
172.9	167.5	162.9	174.0	172.2	171.5	169.62	1.9%	4.4	2.6%
197.6	200.5	209.6	200.0	203.0	196.1	201.84	2.1%	5.0	2.5%

Calculated Concentrations ng/mL

Table 5. Calculated Concentrations of Phosphonic Acids in Urine Donor 2

Spiked Concention Levels of Phosphonic Acids in Donor 2 Urine	Analyis 1 12/4/98	Analysis 2 12/9/98	Analysis 3 12/14/98	Analysisa 4 12/17/98	Analysis 5 12/22/98	Average	Percent Difference Between Calculated and Spiked	STDEV	% RSD
IMPA									
4.82	26.4	5.3	2.7	4.1	0.0	7.70	37.4%	10.6	138.2%
9.64	6.9	14.2	5.9	7.7	5.8	8.10	19.0%	3.5	43.2%
19.3	9.5	18.5	18.9	18.3	23.9	17.82	8.3%	5.2	29.2%
38.6	28.7	38.0	44.3	42.2	40.5	38.74	0.4%	6.1	15.7%
96.4	93.0	87.6	95.7	97.6	104.1	95.60	0.8%	6.1	6.3%
120.5	121.0	120.1	126.0	120.5	119.5	121.42	0.8%	2.6	2.2%
168.7	167.1	175.7	166.4	167.3	164.2	168.14	0.3%	4.4	2.6%
192.8	198.2	191.3	190.9	192.9	193.0	193.26	0.2%	2.9	1.5%
PMPA ISOMER 1									
5.02	7.1	0.0	4.7	19.7	4.6	7.22	30.5%	7.4	103.0%
10.04	11.2	11.6	7.4	5.1	9.6	8.98	11.8%	2.7	30.4%
20.08	20.4	19.7	18.8	14.7	18.7	18.46	8.8%	2.2	12.0%
40.16	37.7	40.8	42.2	34.2	39.3	38.84	3.4%	3.1	8.0%
100.4	95.3	95.5	105.5	100.6	105.8	100.54	0.1%	5.1	5.1%
125.5	117.8	126.8	126.0	125.7	127.3	124.72	0.6%	3.9	3.1%
175.7	207.9	177.8	171.5	174.5	171.3	180.60	2.7%	15.5	8.6%
200.8	180.3	200.4	201.4	203.2	201.2	197.30	1.8%	9.6	4.8%
PMPA ISOMER 2									
5.02	7.1	0.0	4.6	19.9	5.7	7.46	32.7%	7.4	99.8%
10.04	11.3	11.6	7.1	7.0	10.2	9.44	6.4%	2.2	23.8%
20.08	20.4	20.0	18.6	16.2	18.8	18.80	6.8%	1.6	8.7%
40.16	37.5	40.5	43.0	31.6	38.8	38.28	4.9%	4.3	11.1%
100.4	94.5	95.2	105.7	99.5	104.4	99.86	0.5%	5.1	5.1%
125.5	117.8	128.2	126.1	122.8	124.9	123.96	1.2%	4.0	3.2%
175.7	211.1	175.2	171.1	177.1	172.8	181.46	3.2%	16.7	9.2%
200.8	178.0	202.1	201.5	203.6	202.1	197.46	1.7%	10.9	5.5%
CMPA									
4.94	3.7	10.8	6.8	8.8	3.6	6.74	26.7%	3.2	46.8%
9.88	8.8	10.6	11.2	11.1	11.4	10.62	7.0%	1.1	10.0%
19.76	16.9	18.0	18.3	19.5	19.1	18.36	7.6%	1.0	5.5%
39.52	38.4	36.8	39.1	37.2	41.1	38.52	2.6%	1.7	4.4%
98.8	105.9	99.9	100.3	90.3	102.3	99.74	0.9%	5.8	5.8%
123.5	129.8	113.5	119.1	128.6	117.1	121.62	1.5%	7.2	5.9%
172.9	171.7	178.0	169.7	168.2	171.3	171.78	0.7%	3.7	2.2%
197.6	191.8	199.4	202.6	203.1	201.0	199.58	1.0%	4.6	2.3%

Table 6. Calculated Concentrations of Phosphonic Acids in Urine Donor 3

Spiked Concention Levels of Phosphonic Acids in Donor 3 Urine	Analyis 1 12/4/98	Analysis 2 12/9/98	Analysis 3 12/14/98	Analysisa 4 12/17/98	Analysis 5 12/22/98	Average	Percent Difference Between Calculated and Spiked	STDEV	% RSD
IMPA									
4.82	3.5	15.6	4.8	6.0	5.6	7.10	32.1%	4.8	68.3%
9.64	9.2	3.3	10.5	10.4	10.5	8.78	9.8%	3.1	35.4%
19.3	18.8	13.4	18.5	20.2	20.5	18.28	5.6%	2.9	15.7%
38.6	37.7	39.0	38.1	40.0	34.9	37.94	1.7%	1.9	5.1%
96.4	101.4	94.2	98.2	88.8	95.5	95.62	0.8%	4.7	4.9%
120.5	121.0	124.0	119.9	118.9	121.3	121.02	0.4%	1.9	1.6%
168.7	169.3	170.9	165.0	172.6	171.9	169.94	0.7%	3.0	1.8%
192.8	189.7	190.3	195.7	193.7	190.4	191.96	0.4%	2.6	1.4%
PMPA ISOMER 1									
5.02	6.8	0.0	3.8	1.5	6.9	3.80	32.1%	3.1	81.5%
10.04	8.6	0.0	12.5	6.7	10.8	7.72	30.1%	4.8	62.7%
20.08	19.2	9.2	18.4	30.5	20.8	19.62	2.3%	7.6	38.6%
40.16	38.5	72.6	39.1	36.6	37.4	44.84	10.4%	15.5	34.7%
100.4	101.3	118.6	102.8	96.0	99.2	103.58	3.1%	8.8	8.5%
125.5	128.4	120.0	127.2	132.3	123.4	126.26	0.6%	4.7	3.7%
175.7	177.4	171.4	171.7	173.0	179.4	174.58	0.6%	3.6	2.1%
200.8	197.4	194.4	202.4	201.1	199.9	199.04	0.9%	3.2	1.6%
PMPA ISOMER 2									
5.02	3.4	0.0	4.6	1.1	6.4	3.10	61.9%	2.6	83.6%
10.04	9.9	0.0	12.9	6.3	10.4	7.90	27.1%	5.0	63.4%
20.08	20.0	4.7	19.2	32.3	20.7	19.38	3.6%	9.8	50.6%
40.16	38.7	69.2	40.8	36.6	36.8	44.42	9.6%	14.0	31.4%
100.4	104.2	148.6	96.0	98.1	99.3	109.24	8.1%	22.2	20.3%
125.5	128.0	115.6	125.6	126.6	130.2	125.20	0.2%	5.6	4.5%
175.7	177.2	162.7	176.1	175.4	169.8	172.24	2.0%	6.1	3.5%
200.8	196.3	191.1	202.4	201.3	204.1	199.04	0.9%	5.3	2.7%
CMPA									
4.94	0.0	3.5	4.2	3.5	5.9	3.42	44.4%	2.1	62.8%
9.88	11.7	6.9	8.6	9.4	9.8	9.28	6.5%	1.8	18.9%
19.76	15.1	18.5	18.2	18.7	18.4	17.78	11.1%	1.5	8.5%
39.52	34.0	44.4	40.1	38.7	39.1	39.26	0.7%	3.7	9.5%
98.8	115.1	105.4	105.2	112.2	99.5	107.48	8.1%	6.2	5.8%
123.5	121.1	119.6	124.8	116.2	122.7	120.88	2.2%	3.3	2.7%
172.9	170.0	170.2	166.8	168.7	178.1	170.76	1.3%	4.3	2.5%
197.6	195.0	198.4	199.1	199.4	193.4	197.06	0.3%	2.7	1.4%

Calculated Concentrations ng/mL

Table 7. Calculated Concentrations of Phosphonic Acids in Urine Donor 4

Spiked Concention Levels of Phosphonic Acids in Donor 4 Urine	Calculated Concentrations ng/mL					Average	Percent Difference Between Calculated and Spiked	STDEV	% RSD
	Analyis 1 12/4/98	Analysis 2 12/9/98	Analysis 3 12/14/98	Analysisa 4 12/17/98	Analysis 5 12/22/98				
IMPA									
4.82	4.3	5.1	6.1	5.2	4.7	5.08	5.1%	0.7	13.2%
9.64	10.7	9.4	8.4	9.9	11.1	9.90	2.6%	1.1	10.8%
19.3	18.5	19.1	18.8	16.9	19.5	18.56	4.0%	1.0	5.4%
38.6	33.4	36.5	36.5	37.5	37.7	36.32	6.3%	1.7	4.7%
96.4	105.9	99.5	99.9	99.2	97.1	100.32	3.9%	3.3	3.3%
120.5	119.8	121.0	121.5	122.9	117.2	120.48	0.0%	2.1	1.8%
168.7	166.9	169.6	168.2	168.3	169.6	168.52	0.1%	1.1	0.7%
192.8	191.1	190.6	191.3	190.6	193.8	191.48	0.7%	1.3	0.7%
PMPA ISOMER 1									
5.02	5.4	4.4	6.0	5.1	6.3	5.44	7.7%	0.8	13.8%
10.04	10.6	9.6	10.2	9.1	11.6	10.22	1.8%	1.0	9.4%
20.08	20.0	21.9	19.4	18.4	19.9	19.92	0.8%	1.3	6.4%
40.16	36.3	37.6	38.8	38.5	38.5	37.94	5.9%	1.0	2.7%
100.4	106.7	103.1	101.7	107.7	99.3	103.70	3.2%	3.5	3.4%
125.5	123.5	125.4	124.9	126.8	123.2	124.76	0.6%	1.5	1.2%
175.7	174.9	176.7	177.0	171.4	177.7	175.54	0.1%	2.5	1.4%
200.8	200.4	199.0	199.7	200.6	201.3	200.20	0.3%	0.9	0.44%
PMPA ISOMER 2									
5.02	4.7	4.1	6.0	5.1	6.4	5.26	4.6%	0.9	17.9%
10.04	11.0	9.6	11.3	9.3	11.3	10.50	4.4%	1.0	9.3%
20.08	19.9	21.5	18.9	18.4	19.7	19.68	2.0%	1.2	6.0%
40.16	37.4	37.9	37.9	38.1	39.4	38.14	5.3%	0.8	2.0%
100.4	104.4	103.1	104.1	106.0	99.1	103.34	2.8%	2.6	2.5%
125.5	125.2	126.3	123.1	129.3	123.3	125.44	0.0%	2.5	2.0%
175.7	174.7	176.2	173.7	172.2	174.6	174.28	0.8%	1.5	0.8%
200.8	200.4	198.8	202.7	199.3	203.8	201.00	0.1%	2.2	1.1%
CMPA									
4.94	6.7	7.2	5.1	6.1	5.6	6.14	19.5%	0.8	13.7%
9.88	10.5	11.0	9.0	10.1	9.9	10.10	2.2%	0.7	7.4%
19.76	16.7	19.8	18.6	17.3	18.4	18.16	8.8%	1.2	6.6%
39.52	37.1	35.5	42.0	38.2	38.6	38.28	3.2%	2.4	6.3%
98.8	99.4	96.3	101.5	102.0	100.4	99.92	1.1%	2.3	2.3%
123.5	126.6	127.9	119.4	125.9	123.8	124.72	1.0%	3.3	2.7%
172.9	180.1	168.8	172.2	168.3	176.3	173.14	0.1%	5.0	2.9%
197.6	189.8	200.4	199.1	199.1	194.0	196.48	0.6%	4.5	2.3%

Table 8. Calculated Concentrations of Phosphonic Acids in Urine Donor 5

Spiked Concention Levels of Phosphonic Acids in Donor 5 Urine	Calculated Concentrations ng/mL					Average	Percent Difference Between Calculated and Spiked	STDEV	% RSD
	Analyis 1 12/4/98	Analysis 2 12/9/98	Analysis 3 12/14/98	Analysisa 4 12/17/98	Analysis 5 12/22/98				
IMPA									
4.82	5.9	3.8	5.3	3.1	7.0	5.02	4.0%	1.6	31.4%
9.64	12.0	8.9	9.9	7.2	10.6	9.72	0.8%	1.8	18.6%
19.3	17.7	19.6	21.2	18.7	19.3	19.30	0.0%	1.3	6.7%
38.6	36.5	35.8	33.3	47.4	36.8	37.96	1.7%	5.5	14.4%
96.4	92.2	103.8	96.0	94.3	93.6	95.98	0.4%	4.6	4.8%
120.5	123.5	121.3	126.2	116.1	119.3	121.28	0.6%	3.9	3.2%
168.7	174.3	165.9	166.9	170.2	170.6	169.58	0.5%	3.3	2.0%
192.8	188.5	191.6	191.8	193.8	193.5	191.84	0.5%	2.1	1.1%
PMPA ISOMER 1									
5.02	10.2	7.0	5.9	3.7	6.1	6.58	23.7%	2.4	35.9%
10.04	7.6	10.1	10.4	8.7	12.0	9.76	2.9%	1.7	17.2%
20.08	17.0	19.9	20.4	19.2	20.2	19.34	3.8%	1.4	7.2%
40.16	37.2	37.5	36.5	47.0	37.9	39.22	2.4%	4.4	11.2%
100.4	103.7	100.7	102.2	95.9	99.3	100.36	0.0%	3.0	3.0%
125.5	127.4	126.1	127.5	123.7	123.6	125.66	0.1%	1.9	1.5%
175.7	176.0	175.5	173.3	182.9	175.3	176.60	0.5%	3.7	2.1%
200.8	198.6	200.9	201.4	196.7	203.1	200.14	0.3%	2.5	1.3%
PMPA ISOMER 2									
5.02	9.5	6.3	6.3	3.9	5.3	6.26	19.8%	2.1	32.9%
10.04	8.3	9.3	10.6	8.0	9.9	9.22	8.9%	1.1	11.8%
20.08	17.0	19.3	19.4	17.9	19.5	18.62	7.8%	1.1	6.0%
40.16	37.5	37.4	36.8	45.1	36.8	38.72	3.7%	3.6	9.2%
100.4	102.2	103.6	104.1	102.4	101.7	102.80	2.3%	1.0	1.0%
125.5	129.0	128.2	125.7	127.8	130.4	128.22	2.1%	1.7	1.3%
175.7	175.5	173.8	173.0	169.4	179.3	174.20	0.9%	3.6	2.1%
200.8	198.7	199.8	201.9	203.3	194.6	199.66	0.6%	3.3	1.7%
CMPA									
4.94	2.4	6.9	6.5	5.2	3.6	4.92	0.4%	1.9	38.8%
9.88	12.1	12.3	10.9	9.9	12.8	11.60	14.8%	1.2	10.2%
19.76	20.9	18.5	18.8	17.7	18.9	18.96	4.2%	1.2	6.2%
39.52	38.4	43.7	41.8	42.4	38.5	40.96	3.5%	2.4	5.8%
98.8	99.7	93.8	92.3	101.8	107.5	99.02	0.2%	6.2	6.2%
123.5	129.0	111.5	123.1	118.2	112.4	118.84	3.9%	7.4	6.2%
172.9	155.4	177.3	172.3	170.9	172.3	169.64	1.9%	8.3	4.9%
197.6	209.1	202.9	201.1	200.8	200.9	202.96	2.6%	3.5	1.7%

Table 9. Calculated Concentrations of Phosphonic Acids in Urine Donor 6

Spiked Concention Levels of Phosphonic Acids in Donor 6 Urine	Calculated Concentrations ng/mL						Percent Difference Between Calculated and Spiked	STDEV	% RSD
	Analyis 1 12/4/98	Analysis 2 12/9/98	Analysis 3 12/14/98	Analysisa 4 12/17/98	Analysis 5 12/22/98	Average			
IMPA									
4.82	6.1	4.8	2.2	5.8	3.2	4.42	9.0%	1.7	38.0%
9.64	9.7	9.7	17.0	11.9	11.2	11.90	19.0%	3.0	25.3%
19.3	18.3	17.8	15.7	19.4	16.1	17.46	10.5%	1.5	8.8%
38.6	36.8	39.6	37.8	35.3	43.3	38.56	0.1%	3.1	8.0%
96.4	97.0	96.2	94.9	95.5	92.4	95.20	1.3%	1.8	1.8%
120.5	123.3	123.0	120.4	119.6	123.7	122.00	1.2%	1.9	1.5%
168.7	166.3	165.9	170.4	169.6	168.8	168.20	0.3%	2.0	1.2%
192.8	193.3	193.7	192.3	193.5	192.1	192.98	0.1%	0.7	0.4%
PMPA ISOMER 1									
5.02	3.5	16.7	6.5	6.1	5.7	7.70	34.8%	5.2	67.1%
10.04	18.5	6.9	10.4	11.3	15.2	12.46	19.4%	4.5	36.0%
20.08	18.5	17.1	20.0	20.6	18.7	18.98	5.8%	1.4	7.2%
40.16	36.5	38.7	34.1	36.0	38.1	36.68	9.5%	1.8	5.0%
100.4	97.1	86.9	100.6	102.1	94.2	96.18	4.4%	6.0	6.3%
125.5	124.4	130.7	134.5	124.0	126.7	128.06	2.0%	4.5	3.5%
175.7	174.2	177.9	172.9	177.8	175.5	175.66	0.0%	2.2	1.3%
200.8	204.9	202.9	198.6	199.7	203.6	201.94	0.6%	2.7	1.3%
PMPA ISOMER 2									
5.02	3.4	16.7	7.6	5.9	5.7	7.86	36.1%	5.2	65.7%
10.04	20.8	6.5	11.0	11.3	16.2	13.16	23.7%	5.5	41.6%
20.08	17.6	14.8	20.2	20.3	18.5	18.28	9.8%	2.3	12.4%
40.16	35.7	37.0	34.9	36.2	38.0	36.36	10.5%	1.2	3.3%
100.4	96.0	96.6	98.4	102.7	93.7	97.48	3.0%	3.4	3.5%
125.5	123.2	129.6	130.6	124.2	127.1	126.94	1.1%	3.2	2.6%
175.7	177.8	172.8	173.6	177.5	171.5	174.64	0.6%	2.9	1.6%
200.8	203.3	203.7	201.4	199.6	207.1	203.02	1.1%	2.8	1.4%
CMPA									
4.94	2.3	7.8	4.7	6.7	5.5	5.40	8.5%	2.1	38.8%
9.88	8.9	12.2	9.1	12.2	10.3	10.54	6.3%	1.6	15.22%
19.76	15.5	18.4	16.9	18.3	20.5	17.92	10.3%	1.9	10.4%
39.52	40.1	36.6	41.4	40.5	42.5	40.22	1.7%	2.2	5.5%
98.8	111.0	100.9	99.7	92.9	89.5	98.80	0.0%	8.3	8.4%
123.5	132.1	116.5	127.2	122.2	123.0	124.20	0.6%	5.8	4.7%
172.9	153.2	173.9	175.3	172.9	180.4	171.14	1.0%	10.4	6.1%
197.6	203.7	200.6	192.7	201.1	195.3	198.68	0.5%	4.5	2.3%

Table 10. Calculated Concentrations of Phosphonic Acids in Urine Donor 7

Spiked Concention Levels of Phosphonic Acids in Donor 7 Urine	Calculated Concentrations ng/mL					Average	Percent Difference Between Calculated and Spiked	STDEV	% RSD
	Analysis 1 12/4/98	Analysis 2 12/9/98	Analysis 3 12/14/98	Analysisa 4 12/17/98	Analysis 5 12/22/98				
IMPA									
4.82	5.5	0.0	6.6	4.9	6.0	4.60	4.8%	2.6	57.5%
9.64	10.2	7.3	10.8	10.2	9.1	9.52	1.3%	1.4	14.5%
19.3	18.6	17.5	16.7	18.3		17.78	8.6%	0.9	4.8%
38.6	36.9	44.1	38.4	38.2	36.2	38.76	0.4%	3.1	8.1%
96.4	99.0	97.7	96.3	96.0	99.2	97.64	1.3%	1.5	1.5%
120.5	118.9	117.4	119.1	122.7	121.4	119.90	0.5%	2.1	1.8%
168.7	168.9	168.4	170.9	168.7	164.7	168.32	0.2%	2.2	1.3%
192.8	192.7	193.6	192.0	191.8	194.8	192.98	0.1%	1.2	0.6%
PMPA ISOMER 1									
5.02	3.8	5.7	6.7	3.7	5.0	4.98	0.8%	1.3	25.6%
10.04	15.7	10.0	11.3	8.4	9.4	10.96	8.4%	2.9	26.0%
20.08	18.4	18.8	18.6	24.7		20.13	0.2%	3.1	15.2%
40.16	37.3	39.3	39.6	37.8	37.7	38.34	4.7%	1.0	2.7%
100.4	101.9	104.5	97.8	101.0	103.8	101.80	1.4%	2.6	2.6%
125.5	121.9	122.3	126.0	126.6	127.7	124.90	0.5%	2.6	2.1%
175.7	177.9	178.6	176.0	174.7	176.0	176.64	0.5%	1.6	0.9%
200.8	200.9	198.4	201.7	200.8	198.0	199.96	0.4%	1.7	0.8%
PMPA ISOMER 2									
5.02	2.7	5.5	5.4	3.8	4.7	4.42	13.6%	1.2	26.6%
10.04	15.6	10.4	10.4	7.9	9.3	10.72	6.3%	2.9	27.2%
20.08	17.8	19.0	18.5	24.5		19.95	0.7%	3.1	15.4%
40.16	36.1	39.4	39.7	37.5	38.2	38.18	5.2%	1.5	3.8%
100.4	103.6	104.3	101.5	101.0	103.3	102.74	2.3%	1.4	1.4%
125.5	126.1	122.5	127.2	129.1	129.9	126.96	1.1%	2.9	2.3%
175.7	179.5	174.4	175.2	174.2	172.6	175.18	0.3%	2.6	1.5%
200.8	196.4	202.1	199.8	199.8	199.7	199.56	0.6%	2.0	1.0%
CMPA									
4.94	2.8	5.2	3.6	3.9	7.5	4.60	7.4%	1.8	39.9%
9.88	5.3	9.3	7.0	9.6	10.7	8.38	17.9%	2.2	26.1%
19.76	16.2	21.1	21.4	20.2		19.73	0.2%	2.4	12.2%
39.52	33.9	38.9	42.4	36.6	41.4	38.64	2.3%	3.5	9.0%
98.8	122.4	101.0	100.9	97.6	99.6	104.30	5.3%	10.2	9.8%
123.5	132.6	120.5	122.7	133.4	112.3	124.30	0.6%	8.8	7.1%
172.9	163.5	170.1	169.9	173.9	165.2	168.52	2.6%	4.2	2.5%
197.6	190.1	200.8	199.2	191.7	210.5	198.46	0.4%	8.2	4.1%

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Table 11. 5 Day Averages – Urine of Seven Donors

Approximate Spiked Concentration Levels of Phosphonic Acid	Calculated Concentrations ng/mL								
	Water	Urine Donor #1	Urine Donor #2	Urine Donor #3	Urine Donor #4	Urine Donor #5	Urine Donor #6	Urine Donor #7	Average Urine % RSD
IMPA									
5	49.7%	42.5%	138.2%	68.3%	13.2%	31.4%	38.0%	57.5%	55.6%
10	8.6%	17.4%	43.2%	35.4%	10.8%	18.6%	25.3%	14.5%	23.6%
20	12.3%	11.5%	29.2%	15.7%	5.4%	6.7%	8.8%	4.8%	11.7%
40	17.3%	7.6%	15.7%	5.1%	4.7%	14.4%	8.0%	8.1%	9.1%
100	4.1%	2.5%	6.3%	4.9%	3.3%	4.8%	1.8%	1.5%	3.6%
125	1.1%	2.6%	2.2%	1.6%	1.8%	3.2%	1.5%	1.8%	2.1%
175	1.1%	2.2%	2.6%	1.8%	0.7%	2.0%	1.2%	1.3%	1.7%
200	0.8%	1.3%	1.5%	1.4%	0.7%	1.1%	0.4%	0.6%	1.0%
PMPA ISOMER 1									
5	92.4%	19.2%	103.0%	81.5%	13.8%	35.9%	67.1%	25.6%	49.4%
10	46.6%	10.3%	30.4%	62.7%	9.4%	17.2%	36.0%	26.0%	27.4%
20	16.6%	7.0%	12.0%	38.6%	6.4%	7.2%	7.2%	15.2%	13.4%
40	8.2%	4.2%	8.0%	34.7%	2.7%	11.2%	5.0%	2.7%	9.8%
100	11.6%	2.8%	5.1%	8.5%	3.4%	3.0%	6.3%	2.6%	4.5%
125	5.3%	1.8%	3.1%	3.7%	1.2%	1.5%	3.5%	2.1%	2.4%
175	2.4%	1.2%	8.6%	2.1%	1.4%	2.1%	1.3%	0.9%	2.5%
200	2.2%	1.0%	4.8%	1.6%	0.4%	1.3%	1.3%	0.8%	1.6%
PMPA ISOMER 2									
5	110.5%	24.7%	99.8%	83.6%	17.9%	32.9%	65.7%	26.6%	50.2%
10	54.2%	9.7%	23.8%	63.4%	9.3%	11.8%	41.6%	27.2%	26.7%
20	17.5%	9.8%	8.7%	50.6%	6.0%	6.0%	12.4%	15.4%	15.6%
40	9.0%	8.0%	11.1%	31.4%	2.0%	9.2%	3.3%	3.8%	9.8%
100	10.9%	2.7%	5.1%	20.3%	2.5%	1.0%	3.5%	1.4%	5.2%
125	3.5%	1.6%	3.2%	4.5%	2.0%	1.3%	2.6%	2.3%	2.5%
175	2.8%	2.2%	9.2%	3.5%	0.8%	2.1%	1.6%	1.5%	3.0%
200	2.3%	1.0%	5.5%	2.7%	1.1%	1.7%	1.4%	1.0%	2.1%
CMPA									
5	25.6%	15.1%	46.8%	62.8%	13.7%	38.8%	38.8%	39.9%	36.6%
10	9.6%	15.4%	10.0%	18.9%	7.4%	10.2%	15.2%	26.1%	14.7%
20	8.8%	9.6%	5.5%	8.5%	6.6%	6.2%	10.4%	12.2%	8.4%
40	9.9%	8.1%	4.4%	9.5%	6.3%	5.8%	5.5%	9.0%	6.9%
100	4.5%	5.3%	5.8%	5.8%	2.3%	6.2%	8.4%	9.8%	6.2%
125	6.8%	3.1%	5.9%	2.7%	2.7%	6.2%	4.7%	7.1%	4.6%
175	2.2%	2.6%	2.2%	2.5%	2.9%	4.9%	6.1%	2.5%	3.4%
200	1.1%	2.5%	2.3%	1.4%	2.3%	1.7%	2.3%	4.1%	2.4%

Table 12. Relative Percent Differences Between Spiked and Calculated Phosphonic Acid Concentrations
Calculations Performed Using Water Standards, Urine Donor #2 Standards and Urine Donor #4 Standards

Phosphonic Acid Concentration (ng/mL)	Urine Water Stds Run 1	Urine Water Stds Run 2	Urine Water Stds Run 3	Urine Water Stds Run 4	Urine Water Stds Run 5	Average Water Stds	Urine Donor #2 Stds Run 1	Urine Donor #2 Stds Run 2	Urine Donor #2 Stds Run 3	Urine Donor #2 Stds Run 4	Urine Donor #2 Stds Run 5	Average Urine Donor #2 Stds	Urine Donor #4 Stds Run 1	Urine Donor #4 Stds Run2	Urine Donor #4 Stds Run 3	Urine Donor #4 Stds Run 4	Urine Donor #4 Stds Run 5	Average Urine Donor #4 Stds
IMPA																		
4.82	107.5%	678.7%	43.0%	50.8%	10.1%	178.0%	149.7%	701.5%	65.3%	58.5%	100.0%	215.0%	114.0%	664.5%	29.8%	19.7%	24.2%	170.4%
9.64	21.5%	10.3%	45.2%	33.3%	10.6%	24.2%	85.0%	21.8%	53.1%	41.5%	58.5%	52.0%	21.4%	10.0%	29.8%	8.2%	15.0%	16.9%
19.3	13.7%	3.0%	20.4%	21.9%	15.4%	14.9%	61.9%	8.4%	20.1%	18.9%	54.6%	32.7%	7.4%	84.3%	10.2%	4.7%	13.9%	24.1%
38.6	14.4%	6.7%	14.0%	16.9%	13.3%	13.0%	32.9%	9.7%	11.0%	15.2%	53.4%	24.0%	11.3%	6.6%	9.9%	14.8%	13.0%	11.1%
96.4	8.4%	9.9%	7.1%	9.9%	4.8%	8.0%	12.7%	3.2%	3.6%	8.3%	21.6%	11.2%	9.3%	10.2%	5.3%	8.0%	4.9%	7.5%
120.5	8.5%	2.8%	5.7%	7.4%	4.0%	5.7%	11.5%	2.3%	9.1%	6.3%	24.3%	10.9%	5.9%	2.7%	5.6%	5.7%	3.6%	4.7%
168.7	7.6%	3.4%	8.0%	6.0%	2.1%	5.4%	10.4%	3.3%	7.8%	4.7%	21.6%	9.4%	4.9%	3.0%	7.2%	3.4%	3.4%	4.4%
192.8	8.8%	2.4%	5.7%	6.0%	3.1%	5.2%	10.8%		6.1%	5.3%	6.9%	6.5%	6.2%	1.9%	5.3%	3.3%	3.0%	3.9%
IPA ISOMER 1																		
5.02	122.1%	431.8%	36.3%	63.5%	8.7%	132.4%	112.3%	90.8%	34.8%	128.1%	9.3%	75.0%	76.0%	455.4%	48.3%	59.9%	10.6%	130.0%
10.04	42.0%	18.2%	27.7%	11.1%	16.1%	23.0%	37.2%	9.4%	24.6%	59.7%	14.0%	29.0%	25.0%	8.2%	23.2%	3.7%	13.5%	14.7%
20.08	25.9%	13.0%	20.4%	25.9%	24.9%	22.0%	3.7%	6.0%	12.8%	36.0%	30.2%	17.7%	4.5%	7.5%	3.2%	24.1%	18.6%	11.6%
40.16	18.2%	27.0%	12.7%	13.3%	8.4%	15.9%	7.2%	21.7%	7.2%	16.2%	3.6%	11.2%	8.2%	23.4%	6.3%	11.6%	5.3%	11.0%
100.4	5.6%	13.8%	5.6%	6.9%	3.4%	7.1%	5.8%	14.9%	6.3%	7.4%	4.2%	7.7%	3.6%	13.8%	3.7%	5.8%	2.5%	5.9%
125.5	5.5%	6.9%	6.8%	4.1%	3.4%	5.3%	7.3%	3.4%	9.3%	6.8%	3.4%	6.0%	3.5%	1.3%	5.6%	3.6%	1.5%	3.1%
175.7	8.0%	7.1%	7.0%	6.1%	3.6%	6.4%	9.8%	3.2%	6.5%	10.3%	5.0%	6.9%	7.0%	2.7%	6.1%	6.4%	2.8%	5.0%
200.8	5.5%	7.3%	4.7%	5.7%	3.9%	5.4%	9.3%	2.4%	6.9%	6.6%	3.0%	5.6%	4.5%	2.3%	4.0%	2.6%	3.0%	3.3%
IPA ISOMER 2																		
5.02	86.5%	474.3%	41.0%	61.6%	27.1%	138.1%	49.2%	100.7%	16.9%	97.1%	14.7%	55.7%	31.1%	490.4%	44.8%	62.4%	8.7%	127.5%
10.04	58.1%	34.4%	30.2%	9.5%	24.3%	31.3%	45.0%	16.7%	16.4%	33.1%	14.4%	25.1%	33.7%	6.8%	21.5%	4.7%	9.6%	15.3%
20.08	38.5%	21.2%	26.5%	26.5%	33.0%	29.1%	5.0%	6.5%	12.1%	32.3%	28.8%	16.9%	3.2%	8.5%	3.0%	25.7%	26.9%	13.5%
40.16	22.6%	28.1%	15.0%	14.5%	11.7%	18.4%	4.0%	21.1%	6.6%	14.5%	2.3%	9.7%	6.7%	22.6%	6.0%	11.7%	4.4%	10.3%
100.4	5.7%	18.6%	7.3%	6.1%	4.2%	8.3%	4.4%	17.8%	6.6%	5.6%	4.0%	7.7%	4.6%	18.6%	4.2%	5.5%	2.5%	7.1%
125.5	5.4%	4.1%	9.2%	3.9%	3.6%	5.3%	3.5%	3.9%	7.3%	4.4%	3.3%	4.5%	3.8%	0.7%	7.5%	4.2%	2.5%	3.7%
175.7	6.5%	5.3%	7.7%	5.5%	4.4%	5.9%	5.6%	2.0%	3.5%	6.4%	3.1%	4.1%	6.0%	2.8%	4.9%	5.9%	2.7%	4.4%
200.8	5.3%	4.7%	6.6%	5.3%	4.9%	5.3%	4.5%	2.0%	4.7%	2.1%	3.2%	3.3%	5.1%	1.5%	4.3%	4.1%	3.4%	3.7%
CMPA																		
4.94	158.8%	46.9%	109.6%	53.8%	142.6%	102.4%	27.2%	32.6%	23.5%	73.2%	10.5%	33.4%	31.9%	80.5%	15.0%	32.1%	9.9%	33.9%
9.88	65.7%	5.8%	44.7%	19.3%	76.8%	42.5%	21.8%	20.6%	7.1%	27.7%	13.7%	18.2%	15.6%	16.5%	9.7%	8.2%	12.5%	12.5%
19.76	17.1%	14.5%	18.4%	11.0%	39.4%	20.1%	18.5%	9.4%	15.0%	10.3%	11.3%	12.9%	14.5%	7.7%	15.4%	11.8%	14.3%	12.7%
39.52	9.4%	19.8%	13.5%	7.3%	21.5%	14.3%	18.6%	10.1%	9.6%	7.0%	10.5%	11.2%	13.4%	12.1%	7.9%	7.5%	10.6%	10.3%
98.8	18.2%	20.5%	27.6%	11.3%	19.0%	19.3%	46.4%	11.6%	12.4%	14.2%	15.0%	19.9%	17.4%	13.6%	12.7%	10.8%	8.7%	12.6%
123.5	9.7%	22.4%	9.8%	9.1%	12.7%	12.7%	36.8%	13.3%	9.7%	10.5%	12.4%	16.5%	10.3%	13.1%	12.0%	8.1%	13.5%	11.4%
172.9	11.6%	19.1%	11.4%	9.2%	16.9%	13.6%	26.2%	9.7%	9.5%	10.1%	17.1%	14.5%	13.3%	9.7%	11.7%	7.7%	13.8%	11.2%
197.6	7.9%	18.1%	10.3%	11.1%	12.8%	12.0%	32.3%	12.1%	9.2%	11.4%	12.5%	15.5%	9.9%	8.1%	11.1%	10.4%	11.3%	10.2%

25

Table 13. Signal to Noise Ratios for 5 ng/mL Standard

5 ng/mL Standard	Analysis 1 12/4/98	Analysis 2 12/9/98	Analysis 3 12/14/98	Analysis 4 12/17/98	Analysis 5 12/22/98	Average	STDEV	% RSD
IMPA								
Water	21.0	346.0	84.7	55.1	85.6	118.5	129.9	109.7%
Donor #1	63.4	262.8	31.3	193.8	199.2	150.1	98.3	65.5%
Donor #2	543.8	3.2	21.8	200.1	56.6	165.1	225.4	136.5%
Donor #3	5.5	58.8	212.7	168.6	102.2	109.5	83.0	75.8%
Donor #4	60.0	12.0	87.5	59.7	91.5	62.2	31.7	51.0%
Donor #5	7.5	43.8	232.0	43.4	20.4	69.4	92.2	132.8%
Donor #6	33.4	22.4	346.8	33.2	232.8	133.7	148.1	110.8%
Donor #7	30.9	265.5	158.3	26.4	48.4	105.9	104.2	98.4%
					AVERAGE	114.3		
PMPA ISOMER 1								
Water	42.0	114.4	36.1	58.3	30.8	56.3	34.1	60.5%
Donor #1	47.0	90.5	23.1	10.6	55.7	45.4	31.0	68.3%
Donor #2	37.3	65.9	21.0	270.4	45.1	88.0	103.3	117.4%
Donor #3	ND	2.9	124.3	72.8	21.2	55.3	54.7	99.0%
Donor #4	6.2	17.1	148.7	86.5	11.0	53.9	62.3	115.6%
Donor #5	20.5	10.4	18.1	42.9	45.2	27.4	15.7	57.1%
Donor #6	15.1	19.8	120.6	52.7	44.2	50.5	42.3	83.8%
Donor #7	7.9	11.9	57.8	126.0	48.4	50.4	47.6	94.5%
					AVERAGE	53.4		
PMPA ISOMER 2								
Water	50.7	124.2	24.9	51.6	14.7	53.2	42.8	80.5%
Donor #1	58.4	60.5	9.3	30.1	38.5	39.4	21.2	53.9%
Donor #2	55.4	90.3	41.6	188.7	31.9	81.6	63.8	78.2%
Donor #3	ND	3.6	84.3	67.7	16.6	43.0	39.0	90.7%
Donor #4	5.3	16.5	83.9	83.7	33.1	44.5	37.2	83.7%
Donor #5	11.3	9.9	68.8	32.8	36.6	31.9	23.9	75.0%
Donor #6	12.2	26.8	120.6	51.0	39.0	49.9	42.0	84.2%
Donor #7	9.5	14.5	59.6	54.3	46.8	36.9	23.3	63.1%
					AVERAGE	47.5		
CMPA								
Water	11.5	46.2	33.1	88.4	16.7	39.2	30.8	78.5%
Donor #1	30.1	25.7	18.5	51.5	15.6	28.3	14.2	50.2%
Donor #2	24.4	10.8	11.3	11.0	9.8	13.5	6.1	45.6%
Donor #3	ND	8.2	28.9	42.9	19.1	24.8	14.8	59.6%
Donor #4	11.0	31.8	44.1	64.5	62.0	42.7	22.2	52.0%
Donor #5	4.9	7.5	37.6	15.0	7.0	14.4	13.5	94.1%
Donor #6	8.0	11.0	76.1	19.8	47.6	32.5	29.0	89.1%
Donor #7	9.7	21.8	39.2	10.8	28.4	22.0	12.4	56.4%
					AVERAGE	27.2		

Signal to Noise (Peak to Peak)

Table 14. Signal to Noise Ratios for 0.3 and 0.5 ng/mL Standards

	Signal to Noise (Peak to Peak)					
	Analysis 1	Analysis 2	Analysis 3	Average	STDEV	%RSD
0.3 ng/mL Standard						
IMPA						
Water	122.1(carryover?)	6.0	14.1	10.1	5.7	57.0%
Donor #2	31.0	0.1	9.8	13.6	15.8	115.9%
Donor #4	2.0	8.2	11.4	7.2	4.8	66.4%
				AVERAGE 10.3		
PMPA ISOMER 1						
Water	6.9	2.2	8.8	6.0	3.4	56.9%
Donor #2	17.0	2.2	3.1	7.4	8.3	111.6%
Donor #4	2.3	5.1	ND	3.7	2.0	53.5%
				AVERAGE 5.7		
PMPA ISOMER 2						
Water	8.6	2.0	3.3	4.6	3.5	75.5%
Donor #2	17.1	4.0	3.2	8.1	7.8	96.4%
Donor #4	1.8	2.7	ND	2.3	0.6	28.3%
				AVERAGE 5.0		
CMPA						
Water	9.7	3.0	6.3	6.3	3.4	53.0%
Donor #2	6.2	2.9	3.6	4.2	1.7	41.1%
Donor #4	1.9	2.0	0.8	1.6	0.7	42.5%
				AVERAGE 4.0		
0.5 ng/mL Standard						
IMPA						
Water	3.0	3.6	33.8	13.5	17.6	131.0%
Donor #2	4.7	27.2	42.2	24.7	18.9	76.4%
Donor #4	17.7	14.8	29.0	20.5	7.5	36.6%
				AVERAGE 19.6		
PMPA ISOMER 1						
Water	85.8	ND	19.9	52.9	46.6	88.2%
Donor #2	ND	17.6	246.8(?)	17.6	*	*
Donor #4	7.5	13.4	157.3(?)	10.5	4.2	39.9%
				AVERAGE 27.0		
PMPA ISOMER 2						
Water	95.5	ND	8.2	51.9	61.7	119.1%
Donor #2	ND	11.1	225.7(?)	11.1	*	*
Donor #4	7.6	44.1	233.2(?)	25.9	25.8	99.8%
				AVERAGE 29.6		
CMPA						
Water	3.0	0.4	6.6	3.3	3.1	93.4%
Donor #2	1.6	4.6	8.5	4.9	3.4	70.4%
Donor #4	8.3	14.1	6.6	9.7	3.9	40.7%
				AVERAGE 6.0		

Entrys with question marks ere not used in statistics
*Insufficient data touse statistics

APPENDIX A

**Technical Bulletin, TB Med 296, "Assay Techniques for Detection
of Exposure to Sulfur Mustard, Cholinesterase Inhibirors, Sarin, Soman, GF and Cyande"
May, 1996**

TECHNICAL BULLETIN

ASSAY TECHNIQUES

FOR

DETECTION OF EXPOSURE

TO

SULFUR MUSTARD, CHOLINESTERASE

INHIBITORS,

SARIN, SOMAN, GF, AND CYANIDE

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HEADQUARTERS, DEPARTMENT OF THE ARMY

MAY 1996

TECHNICAL BULLETIN }

No. MED 296 }

HEADQUARTERS
DEPARTMENT OF THE ARMY
Washington, DC, 22 May 1996

ASSAY TECHNIQUES FOR DETECTION OF EXPOSURE
TO SULFUR MUSTARD, CHOLINESTERASE INHIBITORS, SARIN,
SOMAN, GF, AND CYANIDE

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CHAPTER 2

VERIFICATION OF SULFUR MUSTARD EXPOSURE—MEASURING THIODIGLYCOL IN URINE BY GAS CHROMATOGRAPH/MASS SPECTROMETER

2-1. Background

Sulfur mustard (HD) exposure can be verified with an assay developed at the Medical Research Institute of Chemical Defense (MRICD).[1] In general, mustard cannot be simply assayed from urine because of its reactive nature. Thiodiglycol (TDG) (2,2'-thiodiethanol) is one of the in vivo degradation products of bis (2-chloroethyl) sulfide (HD)[2,3] and can be used to confirm an exposure. TDG is itself subject to chemical and enzymatic transformations. A recent TDG assay demonstrated the existence of control urines with less than 1 nanogram (ng)/milliliter (ml).[4] However, analyte recoveries were only 50 to 70 percent. In the method presented here, detection of TDG after derivatization with heptafluorobutyric anhydride (HFBA) is achieved by using a gas chromatograph (GC) coupled with a mass selective detector (MSD). The lowest quantifiable concentration is 5.0 ng/ml. Thiodipropanol (TDP) is used as a stabilizer and octa-deuterated thiodiglycol (d_8–TDG) as an internal standard. Through the use of spiked urine standards and the internal standard, a linear regression plot is used to determine TDG concentrations in urine samples.

2-2. Materials and methods

a. Materials. Urine specimens are collected from individuals suspected of exposure to mustard. Control urines should have less than 1 ng/ml TDG before use in standard preparations. Internal standard, d_8–TDG, was obtained from Ash Stevens Incorporated (Detroit, MI). Other materials were obtained commercially. A listing of chemicals and vendors is presented in table 2–1.

b. Supplies and equipment. The procedure requires the use of various pieces of equipment and some common supplies which are listed as follows.

(1) Nitrogen and helium gases, UHP grade (Matheson Gases and Equipment, Montgomeryville, PA).

(2) Polypropylene microcentrifuge tube (Elkay Products Incorporated, Shrewsbury, MA).

(3) N-Evap™ (Organomation Association Incorporated, South Berlin, MA), an evaporator equipped with oil bath and gas nozzles.

(4) Centra-M™ centrifuge (IEC Company, Needham Heights, MA), rotor speed 13,200 revolutions per minute (rpm)/centrifugal force 15,600 × g.

(5) Mixer (Thermolyne Maxi Mix™; Thermolyne Corporation, Dubuque, IA).

(6) GC/MSD, 5890/5970B (Hewlett-Packard, San Fernando, CA).

(7) DB–5 bonded-phase capillary column (J and W Scientific, Folsom, CA), 20 meters, 0.18 millimeter (mm) inner diameter (I.D.), 0.40 micrometer (μm) film thickness.

(8) Pipettes and tips (Rainin Instrument Incorporated, Woburn, MA).

(9) Polyethylene scintillation vials (20 ml capacity).

WARNING

The chemicals involved in these procedures are toxic. Follow all safety precautions listed below.

c. Hazards involved. TDG, d_8–TDG, TDP, and HFBA will be handled in a fume hood. The derivatizing reagent, HFBA, is extremely reactive and toxic. Care must be taken when handling this reagent. Gloves, safety glasses, and a lab coat will be worn when handling chemicals or urine samples. Any further concerns are addressed adequately by the Material Safety Data Sheets.

d. Safety requirements.

(1) *Ventilation.* A general chemical fume hood will be used.

(2) *Clothing.* A standard lab coat, a pair of safety glasses, and latex gloves are required.

(3) *First aid and fire fighting equipment.* There will be standard first aid support and an

[1] Jakubowski, E.M., C.L. Woodard, N.M. Mershon, and T.W. Dolzine. "Quantitation of Thiodiglycol in Urine by Electron Ionization Gas Chromatography-Mass Spectrometry," *J. Chromatog.* 528 (1990), pp. 184–190.

[2] Davison, C.D., R.S. Roman, and P.K. Smith. "Metabolism of Bis-β-Chloroethyl Sulphide (Sulphur Mustard Gas)," *Biochem. Pharmacol.* 7 (1961), pp. 65–74.

[3] Roberts, J.J. and G.P. Warwick. "Studies of the Mode of Action of Alkylating Agents—VI. The Metabolism of Bis-2-Chloroethyl Sulphide (Mustard Gas) and Related Compounds," *Biochem. Pharmacol.* 12 (1963), pp. 1329–1334.

[4] Black, R.M. and R.W. Read. "Detection of Trace Levels of Thiodiglycol in Blood, Plasma, and Urine Using Gas Chromatography-Mass Spectrometry-Electron-Capture Negative Ion Chemical Ionization," *J. Chromatog.* 449 (1988), pp. 261–270.

g. GC/MSD parameters.

(1) The GC parameters are as follows:

(a) The injector port temperature is 220 °C.

(b) The transfer line temperature is 265 °C.

(c) The oven temperature is programmed as: initial temperature is kept at 45 °C for 1.1 minute, increased to 110 °C at a rate of 40 °C/minute, then to 125 °C at 3 °C/minute, and finally increased to 265 °C at 40 °C/minute and kept constant at 250 °C for 1 minute.

(d) The total inlet flow is set at 50 ml/minute.

(e) The split delay time is set at 0.20 minutes.

(f) The column head pressure is set at 12 pounds per square inch.

(g) The septum purge is set at 2 ml/minute.

(2) The MSD parameters are listed below.

(a) Data acquisition is set for selected ion monitoring.

(b) The solvent delay is set at 8 minutes.

(c) From 8.0 to 10.0 minutes, the ions mass to charge ratio (m/z) 300 and 301 representing TDG and m/z 307 and 309 representing d_8 − TDG are monitored. The dwell time for each ion is set at 20 milliseconds (msec), resulting in a cycling time of 7.2 cycles/second.

(d) From 10.0 to 11.0 minutes, the ions m/z 328 and 542 representing TDP are monitored. The dwell time for each ion is set at 20 msec, resulting in a cycling time of 10.0 cycles/second.

h. Analysis of chromatogram.

(1) *Calculations.*

(a) After injecting the samples, the data editor will be used to integrate the peaks in the chromatograph. Retention times of 9.38, 9.42, and 10.5 minutes have been obtained for d_8 − TDG, TDG, and TDP under these conditions.

(b) Integration of the located peak is enhanced by narrowing the time window. When integrating, the threshold is set at 2 or lower for best results. When the integration is complete, the peaks at m/z 300 and m/z 301 will be within 0.04 minutes of the m/z 309 peak.

(c) The area count is used in analyzing the data. The peak area ratio is calculated by dividing the sum of the m/z 300 and m/z 301 areas by the area of the m/z 309 peak. This gives a peak area ratio which when plotted against concentration will yield a positive slope.

(2) *Sensitivity and linearity.* The range of sensitivity for quantitation is from 5.0 ng/ml to 500 ʼg/ml using a standard curve with points plotted at 0, 1, 5, 10, 50, 100, and 500 ng/ml. Linearity is found by using linear regression analysis in Lotus 123™. An r^2 value of at least 0.995 must be obtained before the curve can be used with confidence.

(3) *Precision and accuracy.* This assay was found to be accurate within a range of 5.0 ng/ml to 500 ng/ml. The standard curve is non-linear below 10 ng/ml. Precision varies with concentration ranging from 10 percent relative standard deviation (RSD) (percent RSD = standard deviation (SD)/mean × 100) at 10 ng/ml to 2 percent at 500 ng/ml.

i. Quality control.

(1) Tune the instrument each day prior to use.

(2) Change the septum in the injector port every day.

(3) Inject the samples first and then the standard curve starting with the blank and then proceeding from the lower to the higher concentrations.

(4) The source in the MSD should be cleaned periodically.

(5) Carrier gas should be flowing through the column at all times.

2–3. Results and discussion

a. Derivatization of TDG was necessary for two reasons. First, due to a low molecular weight (122 atomic mass unit) its presence would be obscured by the natural components of urine. Secondly, the polar functional group of TDG causes poor chromatographic peak shape, low sensitivity, and a low relative abundance of molecular ion. Detection limits for underivatized TDG in ethyl acetate were between 500 to 1000 ng on column. TDG can easily be esterified with acyl chlorides other than many anhydrides as was demonstrated by Black and Read.[4] The derivatized esters varied in their stability and usefulness in assays. For example, both the trifluoro and heptafluoro derivatives were prepared and analyzed by GC/mass spectrometer (MS) at MRICD. The HFBA derivative was more stable than the trifluoromethyl anhydride (TFA) derivative. The HFBA derivative produced analytically useful fragments at high molecular weights ranging from m/z 241 to m/z 301. Fragments at m/z 300 and 301 represented the loss of one − $OCOC_3F_7$ group leaving most of the analyte molecule intact. Therefore the identity of the HFBA derivatived analyte was confirmed by the char-

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[4] Black, R.M. and R.W. Read. "Detection of Trace Levels of Thiodiglycol in Blood, Plasma, and Urine Using Gas Chromatography-Mass Spectrometry-Electron-Capture Negative Ion Chemical Ionization," *J. Chromatog.* 449 (1988), pp. 261–270.

Table 2–2. Thiodiglycol levels in rat urine after mustard exposure

HD dose (µg/kg)	TDG concentration (ng/ml)		
	24 hours	48 hours	116 hours
750	196±159	116±76.6	17.2±13.2
450	70.5±19.5	60.6±38.0	6.1±1.4
0	<1	<1	<1

successive steps in the procedure and to recall the results at the end of analyses.

(2) The LED screen displays instructions prompting the user through the analyses procedure and displays the results at the conclusion. In addition, the results are compared to normal mean values preprogrammed in the colorimeter computer and displayed as a percent of the normal mean value.

WARNING

To avoid the transmission of disease, follow all safety precautions listed below.

e. Safety. Precautions must be taken to eliminate hazards either to the operator or the person being tested. The following rules must be followed during the testing procedure:

(1) Do not reuse blood lancets or capillaries.

(2) Put used lancets and capillaries in a hazard bag.

(3) The operator must wear gloves and safety glasses.

f. Erythrocyte AChE activity analyses. Remove the Test-Mate™ components from the carrying case and lay them out, within easy reach, in front of you on a flat surface. In the field it could be the lid of a car trunk, the back of a pickup truck, a flat stone, etc. Before going out in the field, fill the water reservoir bottle with deionized or distilled water and pack an extra battery. Procedures are as follows:

(1) *Press the "Power" key.* The display screen indicates a 15 second warmup countdown.

(2) *Press the "Mode" key.* Press until "AChE mode" is displayed.

(3) *Press the "Test" key.* The display will read "Add buffer" followed by "Insert cuvette" and "Press test." Add four drops of buffer to the cuvette, add water to the 2 ml mark, using the water dispenser, and mix with the stirring paddle. Insert the cuvette into the colorimeter.

(4) *Press the "Test" key.* A 10 second "Blanking" is displayed ending in a "beep." "Remove cuvette" followed by "Press test" is displayed.

(5) *Press the "Test" key.* The display reads "Add blood" followed by "Insert cuvette" and "Press test."

(6) *Wipe the fingertip of the person being tested with the alcohol swab.* Twist off the cap of the blood lancet and prick the finger at its inside tip, off-center. Wipe off the first drop of blood with the gauze pad and squeeze out a second drop. Fill the capillary tube with blood and dispense into the buffer filled cuvette. Mix the solution with the stir-

ring paddle and insert the cuvette into the sample chamber.

(7) *Press the "Test" key.* A 30 second "Reading" is displayed followed by a "beep." During this time Hgb has been determined. The display reads "Remove cuvette" followed by "Press test."

(8) *Press the "Test" key.* The display reads "Get ready" followed by "With reagent" and "Press test." Remove the plastic covering of one of the reagent wells of the microplate with the biopsy punch. Add four drops of water with the water dropper to the well and dissolve the reagent by aspirating it three times using the transfer pipette. Fill the transfer pipette with the dissolved reagent.

(9) *Press the "Test" key.* The display reads "AT BEEP" followed by "Add reagent." At the "beep" add the reagent in the transfer pipette to the diluted blood-filled cuvette and mix the contents with the stirring paddle. The display reads "Insert cuvette" followed by "Press test." Insert the cuvette into the sample chamber.

(10) *Press the "Test" key.* The display shows an 80 second pre-incubation countdown followed by a 50 second reading phase ending in a "beep." The analysis of erythrocyte AChE is concluded. The display reads "Remove cuvette" followed by "Press test." Remove the cuvette and pour the solution into the waste bottle. Use the water dispenser to rinse the cuvette.

(11) *Press the "Test" key to initiate results recovery.* After the first result is displayed, keep pressing the "Disp." key to recover the rest of the data.

g. Plasma BChE activity analyses. Procedures are as follows:

(1) *Press the "Power" key.* The display screen indicates a 15 second warmup countdown.

(2) *Press the "Mode" key until "PChE mode" is displayed.* From this point on, the procedure is the same as for AChE activity determination except that there is no Hgb assay.

3–3. Results and discussion

The Test-Mate™ kit uses blood as sample for both RBC AChE and plasma BChE activity measurement by using two different modes to distinguish the two enzymes. In the RBC AChE procedure, a specific inhibitor of BChE, Astra 1397,[3] has been incorporated into the lyophilized reagent, eliminating interference due to the plasma enzyme. In the plasma BChE activity analysis, the substrate

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[3] Augustinsson, K.B. "A Titrometric Method for the Determination of Plasma and Red Blood Cell Cholinesterase Activity Using Thiocholine Esters as Substrates," *Scan. J. Clin. and Lab. Investigation* 7 (1955), pp. 284–290.

CHAPTER 4

VERIFICATION OF NERVE AGENT EXPOSURE—MEASURING ALKYLMETHYLPHOSPHONIC ACIDS IN URINE BY GAS CHROMATOGRAPH/MASS SPECTROMETER

4-1. Background

In animals exposed to the toxic organophosphorus nerve agents, substantial amounts of the parent compounds are hydrolyzed to their corresponding phosphonic acids, the rest are covalently bound to enzymes and tissue proteins.[1,2,3] Analytical procedures for quantifying the hydrolyzed phosphonic acids in environmental samples have been reported by many analysts.[4,5,6,7,8] For more complex matrices such as biological samples, there has not yet been a method reported to detect these polar acids for verification of exposure. The method described in this chapter is a GC/MS method for the detection of the metabolites of three toxic organophosphorus compounds in urine (sarin, soman, and GF), extracted from a published report.[9] Urinary excretion of the metabolite is the primary elimination route for these three compounds. The major differences among them are

[1] Harris, L.W., L.M. Braswell, J.P. Fleisher, and W.J. Cliff. "Metabolites of Pinacolyl Methylphosphonofluoridate (Soman) after Enzymatic Hydrolysis *in Vitro*," *Biochem. Pharmacol.* 13, (1964), p. 1129.

[2] Reynolds, M.L., P.J. Little, B.F. Thomas, R.B. Bagley, and B.R. Martin. "Relationship Between the Biodisposition of [3H] Soman and its Pharmacological Effects in Mice," *Toxicol. Appl. Pharmacol,* 80, (1985), p. 409.

[3] Lenz, D.E., J. Boisseau, D.M. Maxwell, and E. Heir. "Pharmacokinetics of Soman and its Metabolites in Rats," *Proceedings of the 6th Medical Chemical Defense Bioscience Review,* (1987), p. 201. AD B121516.

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[6] Bossle, P.C., J.J. Martin, E.W. Sarver, and H.Z. Sommer. "High Performance Liquid Chromatography Analysis of Alkyl Methylphosphonic Acids by Derivatization," *J. Chromato.* 267, (1983), p. 209.

[7] Wils, E.R.J. and A.G. Hulst. "Determination of Organophosphorus Acids by Thermospray Liquid Chromatography-Mass Spectrometry," *J. Chromato.* 454, (1988), p. 261.

[8] Tornes, J.A. and B.A. Johnsen. "Gas Chromatographic Determination of Methylphosphonic Acids by Methylation with Trimethylphenylammonium Hydroxide," *J. Chromato.* 467, (1989), p. 129.

[9] Shih, M.L., J.R. Smith, J.D. McMonagle, T.W. Dolzine, and V.C. Gresham. "Detection of Metabolites of Toxic Alkylmethylphosphonates in Biological Samples," *Biol. Mass. Spec.* 20, (1991), p. 717.

primarily the extent and rate of excretion. Nearly total recoveries of the given doses for sarin and GF in metabolite form were obtained from the urine in rats dosed subcutaneously.[10] Soman was excreted at a slower rate with a recovery of only 62 percent. The acid metabolites can be detected in urine for 4 to 7 days after exposure in rats.

4-2. Materials and methods

a. Materials.

(1) Isopropyl methylphosphonic acid (IMPA) and pinacolyl methylphosphonic acid (PMPA) were synthesized by personnel at U.S. Army Chemical Biological Defense Agency, Aberdeen Proving Ground, MD and their methyl deuterated analogs (d₃–IMPA and d₃–PMPA) by Chemsyn Science Laboratories (Lenexa, KS). Cyclohexyl methylphosphonic acid (CMPA) was obtained by hydrolyzing GF in base as described in the literature.[1] Their respective structures are shown in figure 4–1.

COMPOUND	R
IMPA	isopropyl
PMPA	pinacolyl
CMPA	cyclohexyl
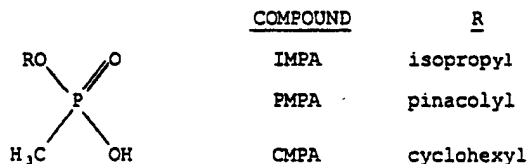
Figure 4–1. Chemical structures of IMPA, PMPA, and CMPA

(2) D₃–PMPA was used as an internal standard for both PMPA and CMPA, and deuterated analog for IMPA. Other chemicals and their respective sources are the following:

(a) Concentrated HCl, powdered anhydrous potassium carbonate, sodium chloride (NaCl) (Fisher Scientific, Pittsburgh, PA).

(b) 100 mg Bond-Elut™ C18 cartridges (Analytichem International, Harbor City, CA).

(c) 1.9 ml microfuge tube (Elkay Products Incorporated, Shrewsbury, MA).

(d) Methanol and methylene chloride (Burdick and Jackson Labs, Muskegon, MI).

(e) 18-crown-6 ether (Eastman Kodak Company, Rochester, NY).

[10] Shih, M.L., J.D. McMonagle, T.W. Dolzine, and V.C. Gresham. "Metabolite Pharmacokinetics of Soman, Sarin, and GF in Rats and Biological Monitoring of Exposure to Toxic Organophosphorus Agents," *J. Appl. Tox.* 14, (1994), p. 195.

dard. The dwell time for each ion is 20 msec resulting in a total scan rate of 6.7 cycles/second.

(2) PMPA is composed of four stereoisomers due to the asymmetric centers at the phosphorus atom and the pinacolyl carbon. Under the chromatographic conditions employed, the stereoisomers are separated into two diastereomer pairs. Quantitation is performed by combining the integrated peak area for each peak. Calibration curves for EI are constructed by plotting the ratio of the peak area of the fragment ion at m/z 256 versus the area of the corresponding m/z 259 fragment ion of the deuterated internal standard. CMPA and IMPA produced only a single peak.

(3) Chemical ionization GC/MS analyses were performed on a Finnegan Incos 50B MS interfaced to a Hewlett-Packard 5890 GC. The GC is fitted with a 30 m × 0.25 mm I.D. DB-5 bonded-phase capillary column, film thickness 0.25 μm. Helium is set at a linear velocity of 37 cm/second (butane injection at 120 °C). The oven temperature is held initially at 40 °C for 2 minutes, programmed from 40 to 200 °C at 20 °C/minute, held at 200 °C for 4 minutes, and then programmed from 200 to 250 °C at 20 °C/minute. Splitless injections of 1 μl are made with the split delay set at 1.5 minutes. Injection port temperature is set at 180 °C; split flow 40 ml/minute; transfer line temperature 240 °C and septum purge 2 ml/minute. The isobutane chemical ionization MS operating conditions are as follows: ion source pressure 1.0 torr; source temperature 115 °C; electron energy 110 eV; and electron emission current 750 μA. The MS is operated in the selected ion mode. In positive CI mode, the MH^+ ion of the derivatized compound is monitored along with its primary fragment ion. In negative CI mode, only the methyl phosphonate anion is observed and monitored (m/z 179 for PMPA, 137 for IMPA, and 177 for CMPA). The scan rate in both positive and negative modes is 0.36 scans/second.

4-3. Results and discussion

a. *Solid phase extraction.* The recovery from solid-phase extraction was determined for PMPA and IMPA using ^{14}C-labeled compounds. From urine samples the values were 94 percent and 85 percent with coefficients of variation (CV) 1.7 percent and 4.5 percent for PMPA and IMPA respectively (N=6). The hydrophobic pi value of the cyclohexyl group is close to the pinacolyl group. Hence, the recovery for CMPA is expected to be similar to PMPA.

b. *Linearity and reproducibility.* In spiked urine samples the peak area ratios were linear over concentration ranges from 10 to 200 ng/ml for PMPA,

IMPA, and CMPA with a correlation coefficient better than 0.99. The coefficient of variation of repetitive assay was less than 5 percent for urine samples (N=6). The lower quantitation limit was 1 ng/ml in urine for PMPA (CV=5.7 percent), 5 ng/ml for CMPA (CV=10.2 percent), and 10 ng/ml for IMPA (CV=9.6 percent).

c. *Chromatograms.*

(1) Table 4-1 shows the retention times of the perfluorobenzyl ester of the various phosphonic acids and the deuterated analogs. The retention time increases proportionately as the lipophilicity of the alkyl side chain of the methylphosphonic acid increases. The PMPAs were resolved into two pairs of diastereomer separated by 0.23 minutes. Control human urine (N=10) did not show any interfering peaks at the region where PMPA and CMPA appeared. An interfering peak did appear at the retention time of IMPA when m/z 256 ion was monitored but not 303 ion. For quantitation purposes a correction from the control sample has to be made if possible. If m/z 256 ion was detected in the EI spectra for unknown samples, CI spectra should be performed to confirm the finding.

(2) The EI mass spectra of all three derivatized acids shared several characteristics. A molecular ion was absent for all three derivatized acids. The base peak for all three compounds was at m/z 181, a non-specific ion of the PFBBr derivatizing reagent. The relative abundance of the major ions observed in the EI mass spectra are summarized in table 4-2. The ready loss of the entire R group under EI conditions produced two fragmentation pathways designated as class specific. Both pathways produced abundant ions for all three compounds. In the first fragmentation scheme the R group was lost along with proton transfer to the remaining oxygen. In addition, protonation of the −P=O oxygen also occurred. This process was observed for both the derivatized PMPA and CMPA and has been reported previously for related organophosphorus compounds.[11,12] Unlike the other two compounds, protonation of just one of the oxygens produced a more prominent ion at m/z 276 for the derivatized IMPA. Further fragmentation with loss of the −PFB (pentafluorobenzyl) group followed by loss of an OH group produced ions at m/z 97 and 80, respectively. The second class specific pathway resulted from the net loss of the R group along with

[11] Occolowitz, J.L. and G.L. White. "The Mass Spectrometry of Esters of Phosphorous and Phosphonic Acids," *Anal. Chem.* 35, (1963), p. 1179.

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Table 4–2. Electron impact mass spectra (Continued)

m/z	Proposed structure	Relative abundance (%)		
		PMPA	IMPA	CMPA
C. Non-specific ions				
181	PFB	100	100	100
161	PFB–HF	8	10	4

Table 4–3. Positive CI mass spectra

m/z	Proposed structure	Relative abundance (%)		
		PMPA	IMPA	CMPA
A. Compound specific ions				
399	[M+C$_3$H$_3$]$^+$ (PMPA)	7	0	0
397	[M+C$_3$H$_3$]$^+$ (CMPA)	0	0	6
361	[M+H]$^+$ (PMPA)	93	0	3
359	[M+H]$^+$ (CMPA)	1	0	100
357	[M+C$_3$H$_3$]$^+$ (IMPA)	0	1	1
319	[M+H]$^+$ (IMPA)	6	100	9
179	[M–PFB+2H]$^+$ (CMPA)	0	1	72
139	[M–PFB+2H]$^+$ (IMPA)	0	23	4
B. Class specific ions				
277	[M–R+2H]$^+$	100	12	65
256	[M–RF]$^+$	4	3	9
C. Non-specific ions				
181	[PFB]$^+$	2	7	9

d. Apparatus. The analytical system consists of the following modules from Technicon Instruments Corporation, Tarrytown, NY.

    (1) Sampler IV.

    (2) Proportioning pump IV.

    (3) Dialyzers, one each, 6-, 12-, and 24-inch lengths with type C membranes.

    (4) Silicon oil heating bath at 116 °C.

    (5) Fluoronephelometer III with Corning Glass filters excitation-#5970 with maximum transmission at 370 nm and emission-#4308 and #3389 with maximum transmission at 470 nm and sharp cut off below 400 nm.

    (6) Flatbed recorder.

    (7) An Eppendorf Model #5412 microcentrifuge.

e. Analyses manifold (fig 5–1).

    (1) The manifold is constructed using the apparatus listed above.

    (2) Standards, plasma, and blood specimens are sampled with one saline wash cup between each at 60/hour resulting in an effective sampling rate of 30/hour. In the free CN^- assay, plasma is mixed with and dialyzed against pH 7.4 phosphate buffer. In the total CN^- assay, the blood is mixed with saline containing Triton X–100 which hemolyzes the erythrocytes and then is acidified with the addition of 0.5 percent sulfuric acid. The sample stream is dialyzed against 0.25 percent sulfuric acid. Additional air is introduced in the recipient stream before distillation.

    (3) The distillation assembly is constructed of glass-to-glass fittings joined with heat shrink tubing. After distillation, the liquid and vapor phases are separated in the manifold trap (#116–0110) and a portion of the latter segments the glycine buffer stream where the CN^- is absorbed.

    (4) An aliquot of the solution is added to the chelate stream where the CN^- demasks the non-fluorescent potassium bis (5-sulfoxino) palladium (II). The resulting 8-hydroxy-5-quiniline sulfonic acid coorditates with magnesium to form the fluorescent chelate.

f. Sample collection.

    (1) Blood should be collected with syringes or Vacutainers™ containing heparin or EDTA. Immediately transfer 250 µl to a 0.5 ml sample cup containing 5 µl of 4–DMAP. Cap, mix and set aside for total CN^- assay. Immediately centrifuge 1 ml of blood for 1 minute at 15,000 rpm, quickly remove plasma and sample for free CN^-. CN^- bound to plasma albumin is unrecoverable.

    (2) After the free CN^- analyses are completed, change to the total CN^- reagents and perform the total CN^- assay on the blood specimens previously set aside.

g. Fluorometer adjustment. All modules are powered up and the manifold filled with reagents.

    (1) Set Sample Aperture to Position C.

    (2) Set Standard Calibration control to 0.00.

    (3) Set Baseline control to 0.00.

    (4) Set Reference Aperture to Position 3.

    (5) Set Function switch to the Reference Position.

    (6) Adjust the light pipe to give a 15 chart units reading on the recorder strip chart.

    (7) Reset Function switch to No Damping.

    (8) Reset Sample and Reference Apertures and Standard Calibration for desired sensitivity (table 5–1).

    (9) Adjust baseline to 0 chart units.

h. Standardization. Standards are prepared by diluting the stock CN^- solution with 0.01 M NaOH. The concentration range of standards should cover the expected CN^- range of the samples. The full set of standards is assayed at the beginning and end of the analyses of the unknowns. An intermediate standard should be sampled periodically as well to provide a measure of precision and correction for gain associated with continuous flow methods.

i. Precision and recovery. Appropriate concentrations of CN^- were prepared by adding µl volumes of stock CN^- to 0.01 M NaOH and to heparinized blood containing 20 µl/ml of 4–DMAP. Each CN^- concentration was analyzed 12 times during one working day.

5–3. Results

a. Standardization. Linear curves are obtained for each range of CN^- aqueous concentrations when µM values are plotted versus fluorescence on cartesian coordinate graph paper. Blood CN^- readings fall within the 95 percent confidence limits of the aqueous standards.

b. Precision. The highest coefficient of variation for aqueous CN^- concentrations ranging from 4000 µM to 1 µM is 5.8 percent and occurs at the lowest concentration measured. Fluorescence units were used to establish the precision in table 5–2.

c. Recovery. The correlation of added versus measured CN^- in blood is excellent (r=0.999). Recovery and other statistical data are presented in table 5–3.

d. Stabilizing total CN^- in blood.

    (1) CN^- added to blood is rapidly bound to Hgb and to plasma proteins. Subsequent analyses show the loss of recoverable CN^- with time.

Table 5–3. Precision and recovery of CN⁻ from blood in vitro

Range	Added CN⁻ (µM)	CN⁻ X	Measured SD	Measured CV%	µM Recovered (%)
I	2500	2511.40	48.08	1.9	100.5
	1000	1063.90	18.04	1.7	106.4
	500	513.30	7.94	1.6	102.7
II	200	202.70	2.57	1.3	101.4
	50	50.50	0.84	1.6	101.0
	20	18.64	0.46	2.7	93.1
III	10	10.12	0.38	3.8	101.2
	5	5.10	0.18	3.5	102.0
	2	2.16	0.05	2.3	108.0

APPENDIX A

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GLOSSARY

Section I
Abbreviations

AChE
acetylcholinesterase

ATCh
acetylthiocholine

BChE
butyrylcholinesterase

BTCh
butyrylthiocholine

C
centigrade

ChE
cholinesterase

CI
chemical ionization

cm
centimeter(s)

CMPA
cyclohexyl methylphosphonic acid

CN-
cyanide

CV
coefficient of variation

dl
deciliter

DTNB
5,5'-dithio-bis (2-nitrobenzoic acid)

d_3-IMPA
deuterated IMPA

d_3-PMPA
deuterated PMPA

d_8-TDG
octa-deuterated thiodiglycol

4-DMAP
4-dimethylaminophenol

EDTA
ethylenediaminetetraacetic acid

EI
electron impact

eV
electron volt

g
gram(s)

GC
gas chromatograph

GF
cyclohexylmethylphosphonofluoridate

HCl
hydrochloric acid

HD
sulphur mustard

HFBA
heptafluorobutyric anhydride

Hgb
hemoglobin

H_2SO_4
sulfuric acid

I.D.
inner diameter

IMPA
isopropyl methylphosphonic acid

kg
kilogram(s)

KH_2PO_4
potassium dihydrophosphate

L
liter(s)

M
mole

mg
milligram(s)

$MgCl_2$
magnesium chloride

ml
milliliter(s)

mm
millimeter(s)
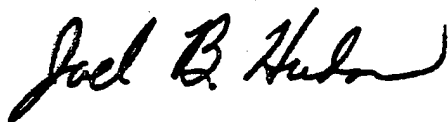
mM
millimole(s)

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APPENDIX B

Recommended Changes to Chapter 2, Technical Bulletin,
TB Med 296, "Assay Techniques for Detection
of Exposure to Sulfur Mustard, Cholinesterase Inhibirors, Sarin, Soman, GF and Cyande"
May, 1996

STUDIES ON AN ANALYTICAL METHOD FOR THIODIGLYCOL

INTRODUCTION

Thiodiglycol, the hydrolysis product of sulfur mustard (SM), has been reported to be a convenient compound to monitor in urine as an indicator for exposure to SM.[1-3] Unfortunately, the assay has limited application because the concentration of urine thiodiglycol decreases rapidly as it is cleared from the body in a few days. Newer methods for indicating or verifying exposure to SM have focused on the analysis of the SM adducts to the amine groups in proteins such as hemoglobin which survives in the body for up to four months after exposure.[4,5] Our original project was to combine the two concepts by measuring the quantities of thiodiglycol liberated by hydrolysis from proteins such as keratin after SM had formed adducts to carboxylic acid sites rather than the amino sites in the molecule. SM esterifies the carboxylic acids and then subsequent hydrolysis of the ester can yield thiodiglycol. Dr. Ming Shih, the scientific coordinator of the project, pointed out that the current procedure for monitoring thiodiglycol was not ideal and measurements at low concentrations tended to show a large degree of variation. Therefore my initial objective for the summer was to reexamine the method for thiodiglycol in the desire to improve the precision and accuracy of the method. However, the study of the thiodiglycol method became the major project for entire summer.

TECHNICAL DISCUSSION

Review of the literature,[1-3] unpublished MRICD studies, and conversations with Dr. Shih and others who have worked on the thiodiglycol method indicated at least nine possible complicating factors that could be associated with the variability in the method. The factors were:

1. Excessive nitrogen and heat drying of the crude sample could lead to loss of thiodiglycol by evaporation,
2. Excessive nitrogen and heat drying of the samples after treatment with the derivatizing agent heptafluorobutyric acid anhydride (HFBAA) could lead to loss of the HFBA-thiodiglycol derivative by evaporation,
3. The use of large excesses of derivatizing agent in a slightly wet solution could lead to high acidity which could slow the reaction, particularly at low concentrations of thiodiglycol,
4. In the presence of excess heptafluorobutyric acid undesirable reactions could occur during high temperature vaporization of the derivative during the initial injection in the chromatographic-mass spectrographic (GC-MS) separation,
5. Excess acid in the reaction mixture could lead to degradation of the HFBA ester derivative of thiodiglycol prior to chromatography,
6. At low concentrations (low nanogram/ml levels of thiodiglycol), the rate of reaction could become much slower than expected,
7. Samples with urine or salt residues were not mixed well enough with the the HFBAA prior to analysis inhibiting formation of the desired product.
8. The reagent, HFBAA, might not be as pure as expected containing high concentration of the acid which could inhibit the reaction, and
9. Both thiodiglycol and the derivative at low concentrations could be lost by interaction with the surface of the containers used in the assay.

Systematic studies of these variables seemed to indicate that the first four factors were more significant than the latter five in leading to large variations in the assay. Complications associated with factors 2, 3, and 4 were clearly exasperated by the excessive quantity of the HFBAA derivatizing agent used as both "drying" agent and derivatizing agent. Improved precision

3.

and **decreased background noise and column contamination** were accomplished by 1. **decreasing the sample drying time to minimize thiodiglycol loss by evaporation, 2. optimizing the quantity of HFBAA derivatizing agent used in the procedure, and 3. filtering the derivatized sample over silica to remove excess heptafluorobutyric acid and HFBAA prior to GC-MS analysis.** The sensitivity of the assay was improved by decreasing loss during drying and the use of a GC-MS with a positive chemical ionization (PCI) detector which enhanced sensitivity about 5 to 10 fold relative to the electron ionization (EI) detector.

A generalized procedure for the analysis is outlined first and then three specific assays, thiodiglycol from ethyl acetate, from urine, and from hydrolyzed albumin are outlined afterwards.

General Procedure:

1. Urine or protein samples were dried by heat and nitrogen gas until they were viscous but still moist appearing. This minimized loss of thiodiglycol by evaporation.
2. Thiodiglycol was extracted with two portions of ethyl acetate solvent. Extracts were combined.
3. The ethyl acetate extracts were dried with sodium sulfate (anhydrous). Note*: sodium sulfate <u>does not</u> absorb all the water in the sample so some excess of derivatizing agent is necessary for optimal reaction.
4. The "dried" ethyl acetate extract was treated with pyridine and a sufficient amount of HFBAA to maximize the yield of derivatized thiodiglycol. Mixing at room temperature for five to 10 minutes appeared to be adequate for derivatization at the 5 nanogram/ml thiodiglycol level.
5. Moist pyridine was added to the solution to hydrolyze any unreacted HFBAA and neutralize the heptafluorobutyric acid formed.
6. The reaction mixture was filtered over a SI (silica) cartridge containing 40 micron silica. About 100 mg of silica were used for each 5 µl of HFBAA used in the derivatizing process (step 4).
7. The eluate was either diluted or concentrated for GC-MS analysis.
8. The SIM mode (ions 301 and 302 for thiodiglycol, and ions 309 and 310 for octadeuterothiodiglycol)in the PCI mode was used for the analysis.
9. Thiodiglycol in the 5 to 10 ng/ml range in the original samples was detected with a reasonable signal to noise ratio. On column concentrations were measured with good signal to noise ratios down to about one pg/µl injected.

Analysis of thiodiglycol added to ethyl acetate:

1. Pyridine (1 µl) and HFBAA (10 µl) were added to ethyl acetate solutions (500 µl) of thiodiglycol at concentrations ranging from 1µg/ml to 10 ng/ml.
2. The mixture was stirred by vortexing for ten minutes.
3. Moist pyridine (10 µl) was added to destroy excess HFBAA and neutralize the HBFA.
4. 250 µl of the reaction mixture (about 1/2 of the total sample) were placed on the SI (silica wet with ethyl acetate) cartridge and pushed through with air. The cartridge was washed with an additional 250 µl of ethyl acetate and that solution added to the first eluate. The volume was adjusted to 500 µl for analysis.
5. One µl of the sample was analyzed by GC-MS. A typical chromatogram is shown in Figure 1. The analyzed sample in Fig. 1 contained the equivalent of 5 pg thiodiglycol (20 pg of thiodiglycol derivative) on column and was derived from a 10 ng/ml thiodiglycol solution. Assays of ≤500 ng/ml to 10 ng/ml thiodiglycol in the original ethyl acetate solutions appeared to be linear with respect to area under the peaks (r^2 = .9981). The coefficient of variation (CV) was 5% at the 10 ng/ml level.

Analysis of thiodiglycol added to urine.

1. The urine sample (one ml) was treated with thiodiglycol and octadeutero-thiodiglycol and evaporated down to a thick but moist mass with a volume of approximately 80 µl. The sample looked and flowed like cold molasses.

2. The urine sample was extracted with a 525 µl portion of ethyl acetate by vortexing rapidly for 10 min. An emulsion formed. Sodium sulfate (about 100 mg) was added to the emulsion and the mixture centrifuged for five minutes. The mixture separated into a clear colorless ethyl acetate layer and a semi-solid pellet. The clear layer was drawn off and the pellet was extracted with a second 525 µl portion of ethyl acetate. A slight emulsion was formed on vortexing for 5 min. and the sample was spun down in the centrifuge again. The mixture again separated into a clear ethyl acetate layer and a light brown pellet. The two extracts were combined, adjusted to a volume of 1 ml, and treated with sodium sulfate (about 100 mg).

3. 250 µl of the combined "dried" extracts were treated with pyridine (3 µl) and HFBAA (30 µl) and vortexed for 15 minutes. (Note*: reactions with 20 µl and 40 µl of HFBAA were also performed, but the 30 µl reaction was optimal).

4. Moist pyridine (30 µl) was added to the solution.

5. A 50 µl portion was placed on the SI cartridge and eluted with 450 µl of ethyl acetate.

6. The eluate was either analyzed by GC-MS directly or after concentration by evaporation. Fig. 2 shows results of the analysis of a sample of urine (one ml) containing 100 ng of octadeuterothiodiglycol and 10 ng of thiodiglycol. The quantities of the analytes on column equivalent to 3.4 pg of deuterated thiodiglycol and 0.35 pg thiodiglycol. The expected quantities were 5.0 and 0.5 pg, respectively, so the recovery was 68%. Fig. 3 shows the results of the urine assay starting with 25 ng/ml of deuterated thiodiglycol and 5 ng/ml thiodiglycol in one ml of urine. With concentration of the sample after silica filtration, the equivalent deuterated thiodiglycol concentration should have been 28 pg on column but 17.5 pg was measured. The quantity of thiodiglycol measured was 3.5 pg on column instead of 5.6 pg. Therefore the recovery was 62%.

Analysis of thiodiglycol obtained from albumin treated with SM:

1. A sample (4 mg) of albumin previously treated with aqueous SM (2.5 mg SM and 60 mg BSA in 5 ml saline vortexed for 2 hr at room temperature, precipitated with acetone and washed with water) was dissolved in NaOH (1.0 M, .5 ml) and heated in an oil bath at 70°C for 2hr.

2. After cooling, the sample was acidified to pH 5.0 (indicator paper) by addition of 1.0 M HCl.

3. The acidified sample was then evaporated down to a mass of material stuck to the side of the vial.

4. The sample was extracted and centrifuged twice with ethyl acetate (.5 ml).

5. The combined extracts (one ml) were dried with sodium sulfate (about 200 mg.)

6. 250 µl of the extract was diluted to 500 µl and was then treated with pyridine (2µl) and HFBAA (20 µl) and vortexed for 15 minutes.

7. Moist pyridine (20 µl) was then added to the mixture and 100 µl of the solution placed on a 100 mg SI cartridge. The cartridge was washed with 400 µl of ethyl acetate and the total volume adjusted to 500 µl.

8. The eluate was analyzed by GC-MS. Three samples of slightly different masses of albumin showed almost identical quantitation of about 300 pg/µl or 300 ng/ml. Fig. 4 is an example of one of the assays and Fig. 5 is an analysis of a control to ensure that all unreacted SM and unbound thiodiglycol had been washed from the protein sample prior to treatment with base. Based on the analysis, it is estimated that on the average one

sulfur mustard molecule reacted at a free carboxyl group with one out of ten albumen molecules.

Reactions of thiodiglycol with pentafluorobenzoyl chloride:

Pentafluorobenzoyl chloride, PFBCl, has been reported to be an excellent derivatizing agent for thiodiglycol, giving a product detectable in the low femtogram range.[2] A comparison of the two derivatizing reagents, HFBAA and PFBCl seemed appropriate so the derivative of thiodiglycol was prepared and reagent was tested with thiodiglycol in the derivatization reaction. Indeed the pentafluorobenzoyl thiodiglycol derivative could be measured down to the low femtogram range and the reaction process with thiodiglycol in ethyl acetate occurred readily as with HFBAA. Unfortunately the summer ended and a more detailed study was not attempted.

Conditions for the GC-MS analysis:

The PCI GC-MS method for the analysis of HFBA thiodiglycol derivatives included an inlet temperature of 250°C, source temperature of 250°C, an initial column temperature of 60°C for two minutes and then a temperature ramp of 25°C/min until the column temperature was 180°C. The retention time for the thiodiglycol derivative was 6.67 min and 6.65 min for the octadeuterothiodiglycol derivative. An HP5 column was used.
Conditions for the NCI analysis of the PFBA derivative of thiodiglycol that were changed included a source temperature of 150°C, an initial column temperature of 90°C and a 30°/min ramp to 215°C. The retention time was 12.5 minutes.

CONCLUSIONS

A systematic study of the variables associated with a GC-MS method for the analysis of thiodiglycol led to improvement in the precision and sensitivity of the method particularly at the low nanogram/ml levels of thiodiglycol. The four modifications were:

1. Decreased drying of the thiodiglycol sample so that loss of thiodiglycol by evaporation was minimized,
2. Optimizing the quantity of the HFBAA derivatizing agent used so that a minimum of post-reaction sample clean-up was necessary,
3. Filtering the derivatized sample over a silica (SI) cartridge to remove excess HFBAA and heptafluorobutyric acid prior to GC-MS analysis, and
4. Using a more sensitive positive chemical ionization detector in the single ion monitoring (SIM) mode rather than an electron ionization (EI) detector in the SIM mode.

These four modifications of the procedure should facilitate subsequent studies on monitoring the magnitude of exposure of proteins to sulfur mustard.

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2. R. M. Black and Robert W. Read, Detection of Trace Levels of Thiodiglycol in Blood, Plasma and Urine Using Gas Chromatography-Electron-Capture Negative-Ion Chemical Ionisation Mass Spectrometry, *J. Chromatogr.*, **449**, 261-270, 1988.
3. E. M. Jakubowski, C. L. Woodard, M. M. Mershon, and T. W. Dolzine, Quantification of thiodiglycol in urine by electron ionization gas chromatography-mass spectrometry, *J. Chromatogr.*, **528**, 184-190, 1990.
4. A. Fidder, D. Noort, A. L. de Jong, H. C. Trap, L. P. A. de Jong, and H. P. Benschop, Monitoring of in Vitro and in Vivo Exposure to Sulfur Mustard by GC/MS Determination of the N-Terminal Valine Adduct in Hemoglobin after a Modified Edman Degradation, *Chem. Res. Toxicol*, **9**, 788-792, 1996.
5. R. M. Black, R. J. Clarke, J. M. Harrison, and R. W. Read, Biological fate of sulphur mustard: identification of valine and histidine adducts in haemoglobin from casualties of sulphur mustard poisoning, *Xenobiotica*, **27**, 499-512, 1997.
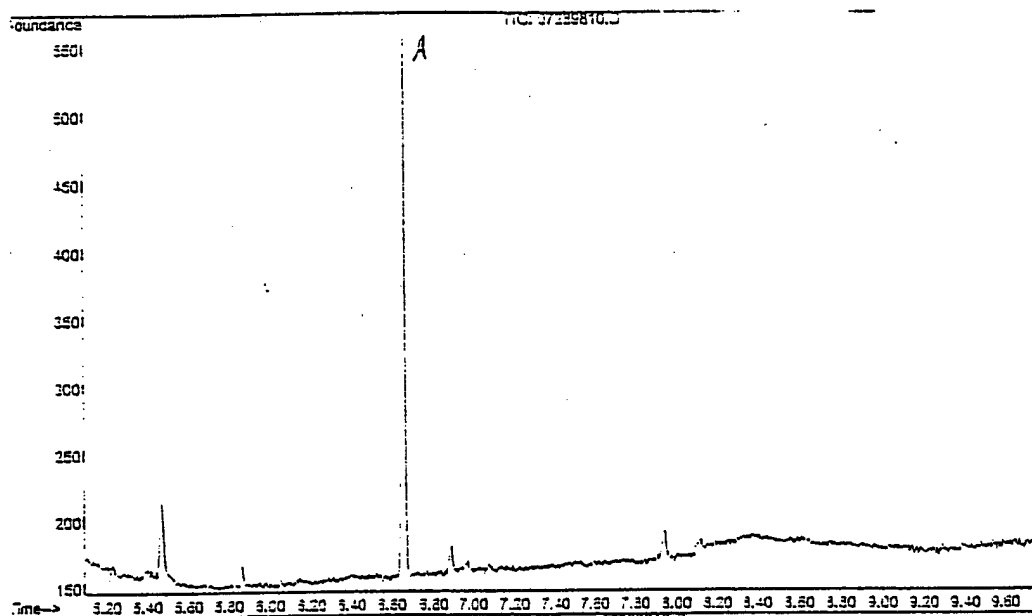
Fig. 1. Chromatogram of the solution from the formation of the HFBA derivative of thiodiglycol in a 10 ng/ml ethyl acetate solution. Peak A represents the equivalent of approximately 5 pg thiodiglycol.
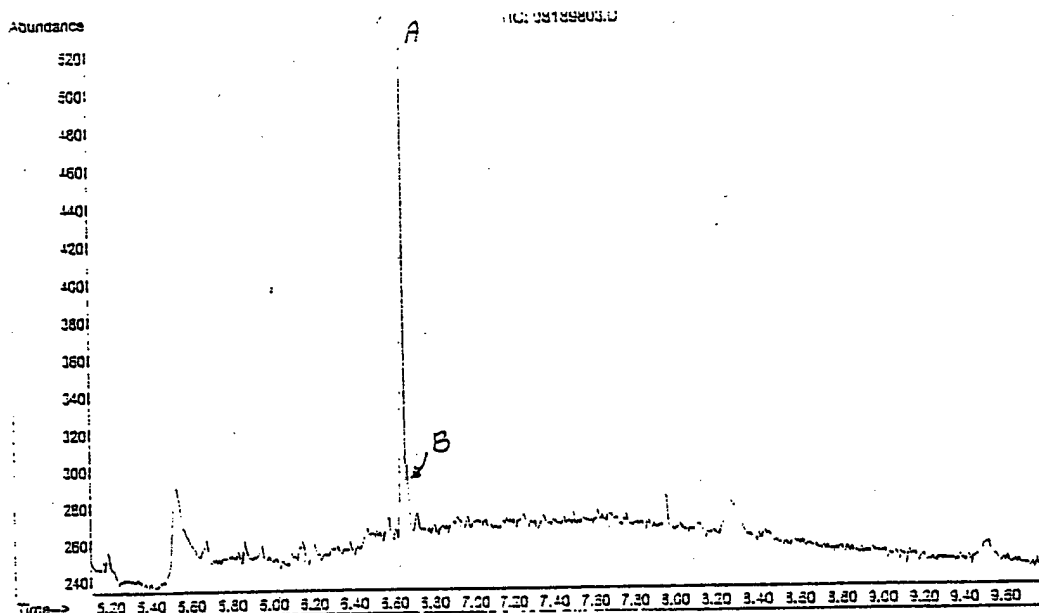
Fig. 2. Chromatogram of the solution from the formation of the HFBA derivative
of thiodiglycol extracted from a 10 ng/ml urine sample. Peak A
represents the equivalent of 3.4 pg of octadeuterothiodiglycol
and peak B represents the equivalent of .34 pg of thiodiglycol.
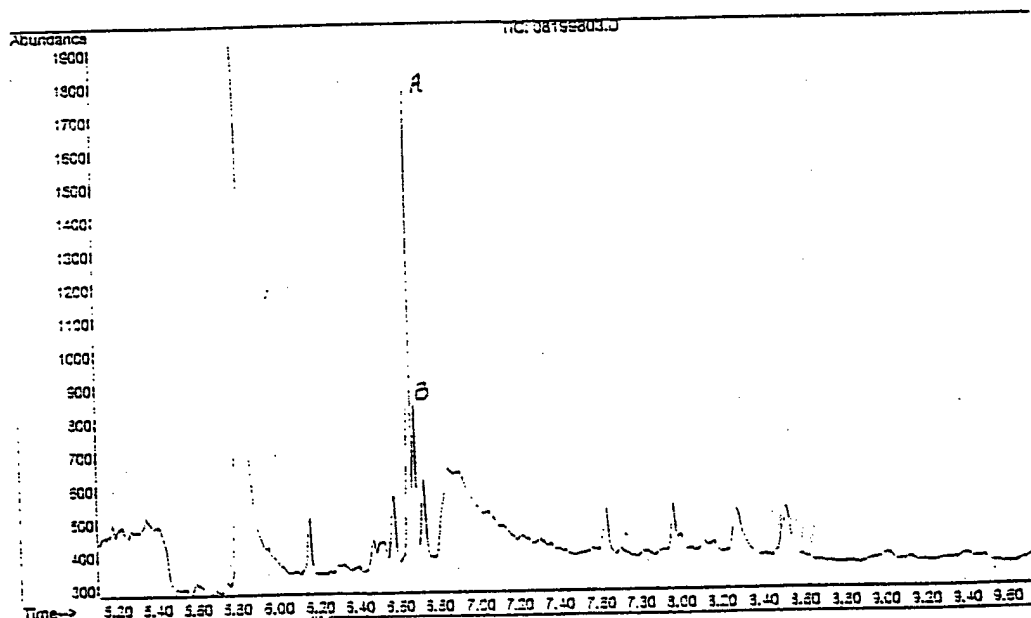
Fig. 3. Chromatogram of the solution from the formation of the HFBA derivative
of thiodiglycol extracted from a 5ng/ml urine sample. Peak A is the
equivalent of 17.5 pg. octadeuterothiodiglycol and peak B is the
equivalent of 3.5 pg of thiodiglycol.

9.
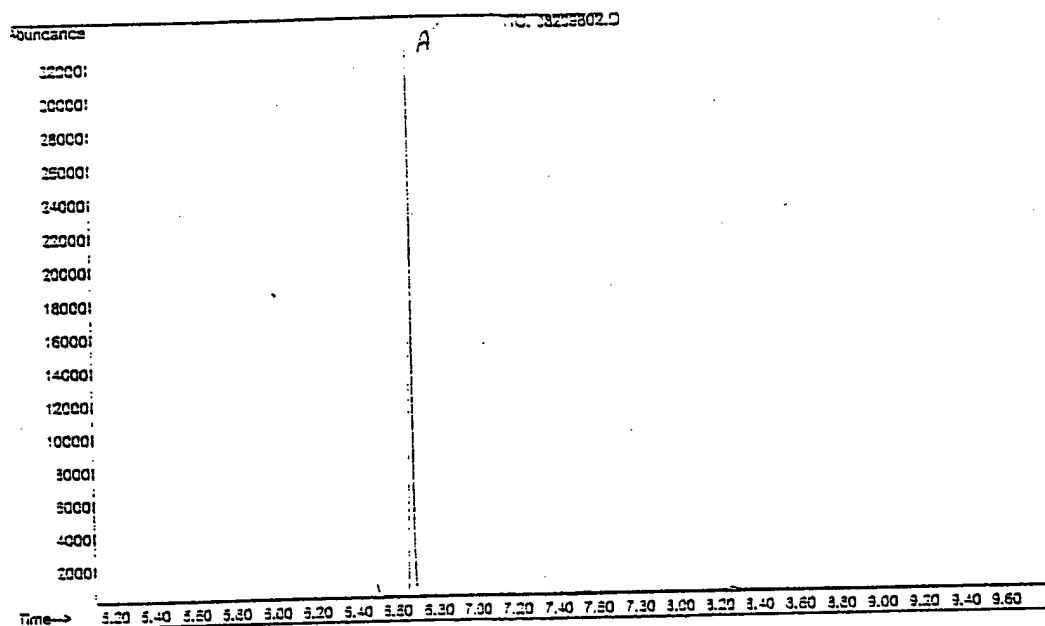
Figure 4. Chromatogram of the solution containing the HFBA derivative
of thiodiglycol from the albumen reaction. Peak A represents
the equivalent of 300 pg of thiodiglycol.
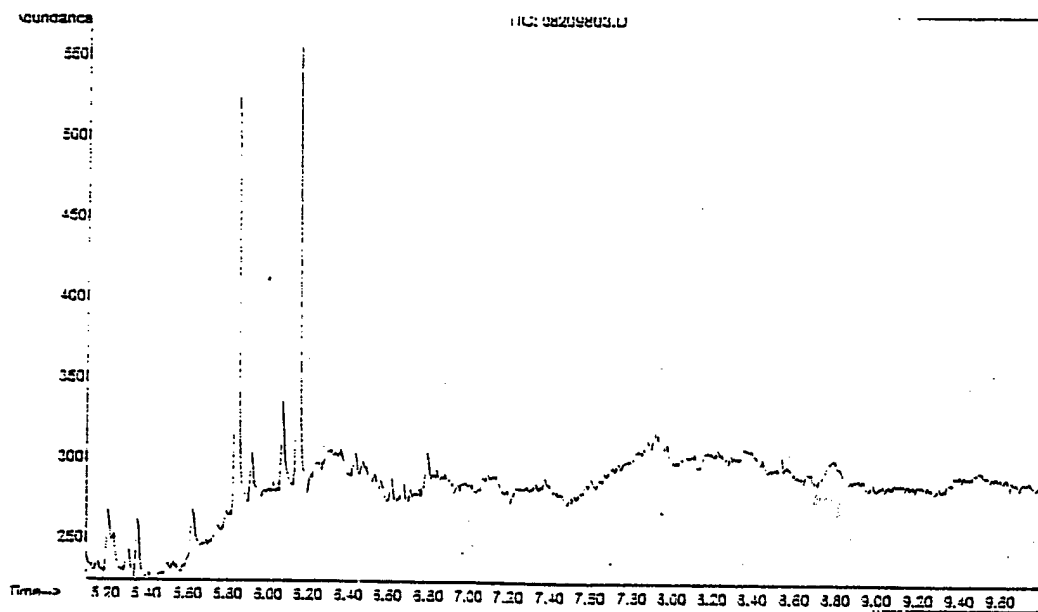
Fig. 5. Chromatogram of HFBA treated control solution. Note the absence
of significant peak at 6.65 min.

10.

APPENDIX C

Method No. 18/Chemistry
Method for Verification of Sulfur Mustard Exposure – Measuring Thiodiglycol in Urine
by Gas Chromatograph/Mass Spectrometer

METHOD FOR VERIFICATION OF SULFUR MUSTARD EXPOSURE – MEASURING THIODIGLYCOL IN URINE BY GAS CHROMATOGRAPH/MASS SPECTROMETER

A. Statement of Work:

This method describes the detection of thiodiglycol (TDG) standard after derivatization with heptafluorobutyric acid anhydride (HFBAA) by gas chromatography (GC) coupled with mass spectrometry (MS). Based on data from the precision and accuracy study, the lowest detectable concentration is approximately 5.0 ng/mL. The quantitation range is from 15 -25 ng/mL to 500 ng/mL of TDG with the lower quantitation level being highly dependent upon the matrix. Through the use of spiked urine standards and the internal standard, a linear regression plot is used to determine TDG concentrations in urine samples.

B. Purpose:

In general, sulfur mustard (HD) cannot be simply assayed from urine because of its reactive nature. Thiodiglycol (TDG – 2,2'-thiodiethanol) is one of the *in vivo* degradation products of bis (2-chloroethyl) sulfide (HD) in both animals and human beings and can be used to confirm an exposure. TDG is itself subject to chemical and enzymatic transformations.

C. Samples:

This method is being validated for use in the analysis of both aqueous and human urine samples. At concentration levels below 25 ng/mL, the concentrations of TDG in urine should be determined using pre-exposure urine from the person involved or overspiking of the post-exposure urine with TDG. At levels above 25 ng/mL, it is acceptable to use pooled urine spiked with TDG for calibration, however, high purity water may be used when urine is not available.

D. Materials: (equivalent items may be substituted)

1. Thiodiglycol-d$_8$ (Material #: 98R-003) [CAS not determined] was prepared by Dr. Allison Fentiman, Battelle Memorial Institute, 505 King Ave., Columbus, Ohio 43201.

2. 2,2'-thiodiethanol, 99%, Aldrich Chemical Co., catalog number 16,678-2, [CAS 111-48-8].

3. Molecular sieves (5A), Sigma Chemical Co., catalog number M-1510.

4. Heptafluorobutyric anhydride (HFBAA), Pierce Chemical Co., catalog number 63164, [CAS 336-59-4]. (NOTE: HFBAA should be purchased in 1 mL sealed ampoules since it is susceptible to decomposition by water in the atmosphere.)

5. Water, Baker Instra Analyzed, VWR, catalog number JT4219-3, [CAS 7732-18-5].

6. Benzene, OmniSolv, BX0212-3, [CAS 71-43-2].

7. Pyridine, Burdick and Jackson, UN1282, [CAS 110-86-1].

8. Sodium sulfate, EM Tracepur, SX0760E-3, [CAS 7757-826-6].

9. Methanol, Burdick and Jackson, UN 1230, [CAS 67-56-1].

E. **Equipment: (equivalent items may be substituted)**

1. Nitrogen and helium gases, 99.999% pure.

2. Polypropylene microcentrifuge tubes (w/racks), 2 mL, VWR, catalog number 53550-790.

3. Reacti-Therm III (Pierce, Rockford, IL, 18935) an evaporator equipped with aluminum blocks (Reacti-Block, V-1) and gas nozzles (Reacti-Vap III, 18785).

4. Centrifuge, Eppendorf Centrifuge 5414.

5. Vortex mixer, VWR, Vortexer 2.

6. Haake Buchler Vortex Evaporator.

7. GC/MS, Micromass, Quattro II with an electron impact source.

8. HP-5MS bonded-phase capillary column (Hewlett Packard, Wilmington, DE, 19091S-431), 15 meters, 0.25 mm inner diameter, 0.25 μm film thickness.

9. Pipettors and tips, air displacement, ranges 10-100 μL, 20-200 μL, 100-1000 μL.

10. Glass scintillation vials (20 mL capacity), VWR, catalog number 66021-726.

11. pH paper, pHydrion, pHydracid 1-6, VWR, catalog number 60777-027.

12. Volumetric flasks, 10 and 25 mL capacity, Class A.

13. Pipets, 1, 5, 10 mL, Class A.

14. Glass syringes: 10 μL, 25 μL, 50 μL, 250 μL, 500 μL, 1000 μL and 1.5 mL capacity.

15. 100 mg BondElut, Si cartridges, Varian/Rainin, catalog number 1210-2010.

16. 5 mL disposable syringe with adapter to fit BondElut cartridges.

17. Solid phase extraction vacuum manifold/collection system, Supelco, 5-7030.

F. **Methodology For Urine Collection:**

The method of urine collection will be defined in the protocol; however, it is imperative that the samples are well mixed and representative.

Store the urine samples in plastic containers, labeled with the source and/or laboratory record book number and date. Freeze the samples until needed for analysis. It is advantageous to divide a single sample between smaller containers with aliquots defrosted as needed. This will limit the number of freeze/thaw cycles necessary. Expiration date of the urine samples will be one year from the date of collection.

G. **Preparation of the standards:** (record all weights in a numbered laboratory record book, equivalent dilutions may be made)

1. Preparation of the stock solutions:

   a) *1 mg/mL Stock TDG.* Into a 25 mL volumetric flask weigh out approximately 25 ± 5 milligrams (mg) of TDG, dilute to volume with methanol and vortex to mix. Transfer this solution to a glass scintillation vial and label with the solution identification (1 mg/mL *Stock TDG)*, the concentration in mg/mL, the preparation and expiration dates, laboratory record book number and page and the name of the preparer. This solution should be stored under refrigeration (0 –10 °C). Prepare fresh solution at least monthly.

   b) *1 mg/mL Stock D_8-TDG.* Into a 25 mL volumetric flask weigh out approximately 25 ± 5 milligrams (mg) of d_8-TDG, dilute to volume with methanol and vortex to mix. Transfer this solution to a glass scintillation vial and label with the solution identification (1 mg/mL *Stock D_8-TDG)*, the concentration in mg/mL, the preparation and expiration dates, laboratory record book number and page and the name of the preparer. This solution should be stored under refrigeration (0 –10 °C). Prepare fresh solution at least monthly.

c) *5 µg/mL Stock TDG.* Using a syringe, transfer 50 µL of the *1 mg/mL Stock TDG* into a 10 mL volumetric flask containing approximately 5 mL methanol. Dilute to volume with methanol and vortex to mix. Transfer the solution to a glass scintillation vial and label with the solution identification (*5 µg/mL TDG*), the concentration in µg/mL, the preparation and expiration dates, laboratory record book number and page and the name of the preparer. This solution should be stored under refrigeration (0 –10 °C). Prepare fresh solution at least monthly.

d) *5 µg/mL Stock D$_8$-TDG.* Using a syringe, transfer 50 µL of the *1 mg/mL Stock D$_8$-TDG* into a 10 mL volumetric flask containing approximately 5 mL methanol. Dilute to volume with methanol and vortex to mix. Transfer the solution to a glass scintillation vial and label with the solution identification (*5 µg/mL D$_8$-TDG*), the concentration in µg/mL, the preparation and expiration dates, laboratory record book number and page and the name of the preparer. This solution should be stored under refrigeration (0-10 °C). Prepare fresh solution at least monthly.

2. Preparation of the working standards:

a) *1 µg/mL Working TDG Standard.* Using a syringe, transfer 2 mL of *5 µg/mL Stock TDG* solution to a 10 mL volumetric flask containing approximately 5 mL of methanol. Dilute to volume with methanol and vortex to mix. Transfer this solution to a glass scintillation vial and label with the solution identification (*1 µg/mL Working TDG Standard*), the concentration in µg/mL, the preparation and expiration dates, laboratory record book number and page and the name of the preparer. Prepare this solution fresh weekly and store under refrigeration (0 –10 °C).

H. Analytical Method:

THIS METHOD IS HIGHLY MATRIX DEPENDENT. AT CONCENTRATION LEVELS ABOVE 25 NG/ML, WATER MAY BE USED AS THE MATRIX FOR STANDARDS PREPARATION IF THERE IS NO OTHER OPTION, HOWEVER, URINE (PREFERABLY PRE-EXPOSURE URINE OF THE PATIENT) IS PREFERRED.

FOR DETEMINATIONS AT CONCENTRATION LEVELS BELOW 25 NG/ML, IT IS NECESSARY THAT PRE-EXPOSURE URINE FROM THE PATIENT BE USED FOR STANDARDS PREPARATION. IF THIS IS NOT AVAILABLE, THE POST-EXPOSURE URINE CAN BE OVERSPIKED WITH TDG AND THE CONCENTRATION BACK-CALCULATED.

1. Preparation of sample/standard microcentrifuge tubes:

   a) A minimum of three standard concentration levels plus a blank are necessary for the analysis, five levels are recommended. In all cases, the concentrations of the standards must bracket the concentrations of the actual samples. Label the microcentrifuge tubes that are to be used to contain the standards (0 - 250 ng/mL). Into each of the tubes pipet 1.0 mL of Instra Analyzed water or pre-exposure urine.

   b) Using the appropriate size syringe, add 0 - 250 µl of the *1 µg/mL Working TDG Standard* to tubes labeled in step a. Vortex each tube.

   c) Using the appropriate size syringe, add 20 µL of the *5 µg/mL Stock D_8-TDG Standard* to each of the microcentrifuge tubes. Vortex each tube.

   d) For the analysis of the urine of actual HD exposed victims (concentrations > 25 ng/mL), add 1.0 mL of urine to microcentrifuge tubes as in step a. Do not spike with TDG. Continue with step H.1.c. It is recommended that these urine samples be analyzed in triplicate.

   e) For the analysis of the urine of actual HD exposed victims (concentrations < 25 ng/mL) where pre-exposure urine is not available, spike 1.0 mL of the sample urine with at least three concentration levels of TDG. Continue with step H.1.c.

2. Sample Extraction:

   a) Place all microcentrifuge tubes in the Reacti-Therm III set between 70 °C and 75 °C and evaporate the solutions down to a volume of approximately 80 µL under a gentle flow of nitrogen. This normally takes approximately 1.5 hours.

   b) Remove the tubes from the Reacti-Therm and allow them to cool to room temperature.

   c) Using a syringe, add 500 µL of benzene to each tube and vortex each vigorously.

   d) Add approximately 200 mg of finely ground sodium sulfate to each tube and vortex. (Note: the sodium sulfate must be heated overnight at 400 °C to remove impurities, then cooled, ground to a fine powder and stored in a 110 °C-130 °C oven when not in use).

   e) Vortex all microcentrifuge tubes for approximately 10 minutes. Use of an automatic vortexer such as the Haake Buchler Vortex Evaporator is acceptable.

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    d) For the analysis of the urine of actual HD exposed victims (concentrations > 25 ng/mL), add 1.0 mL of urine to microcentrifuge tubes as in step a. Do not spike with TDG. Continue with step H.1.c. It is recommended that these urine samples be analyzed in triplicate.

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    e) Vortex all microcentrifuge tubes for approximately 10 minutes. Use of an automatic vortexer such as the Haake Buchler Vortex Evaporator is acceptable.

f) Centrifuge for 1 minute. At this point, it may be necessary to add additional sodium sulfate to tubes which still contain a free water layer between the sodium sulfate and benzene layer. After the addition, repeat vortexing (1 minute) and centrifuging (1 minute).

g) Using a syringe, remove the benzene layers and place in identically labeled clean microcentrifuge tubes.

h) Add an additional 500 µL of benzene to each of the original tubes and vortex vigorously (approximately 30 seconds). Centrifuge each tube for 1 minute.

i) Using a syringe, transfer the second benzene extract to the matching tube containing the first extract. Discard the initial microcentrifuge tubes.

j) Add approximately 100 mg of sodium sulfate to the tubes containing the benzene extracts. Vortex (approximately 30 seconds) and centrifuge (approximately 1 minute). The sodium sulfate should at this point be free flowing. If it clumps there is still water in the benzene. Add additional sodium sulfate to the tube in question, followed by vortexing and centrifuging.

k) Transfer the dried benzene extracts to clean identically labeled microcentrifuge tubes.

l) Add 30 µL of HFBAA to each tube, cap immediately and vortex.

m) Next add 3 µL of dry pyridine (pyridine stored over molecular sieves) to each tube, cap immediately and vortex.

n) Place all microcentrifuge tubes in the Reacti-Therm set at 50 °C for 15 minutes.

o) Prepare the appropriate number of Si cartridges (one for each microcentrifuge tube) by washing each with 1 mL of benzene. Gently force all the benzene through the cartridges using a plastic syringe with adapter.

p) Using a disposable plastic pipet, add the benzene solution in the microcentrifuge tube to a Si cartridge and elute into a labeled GC vial using the plastic syringe with adapter. The rate of elution must be no more than approximately one drop per second, not only to ensure sufficient clean up of the sample, but also to prevent the eluant from splashing out of the GC vial. Cap each vial.

q) Inject 1 to 2 µL of the extract into the GC/MS.

I. GC/MS parameters:

1. The following GC parameters are considered recommended starting conditions. The actual conditions will be optimized by the operator.

   a) Column - HP-5MS bonded-phase capillary column (Hewlett Packard, Wilmington, DE, 19091S-431), 15 - 30 meters, 0.25 mm inner diameter, 0.25 μm film thickness.

   b) The injector port temperature is 220 ± 10 °C.

   c) The transfer line temperature is 280 ± 10 °C.

   d) Source temperature is 250 ± 10 °C.

   e) The oven temperature is programmed as follows: initial temperature is kept at 60 °C for 2 minutes, increased to 110 °C at a rate of 40 °C/minute, then programmed to 125 °C at 3 °C/minute, and finally increased to 300 °C at 49 °C/ minute and kept constant at 300 °C for 2 minutes.

   f) The linear velocity with helium is approximately 35 cm/sec at 100 °C using freon.

   g) The split flow is at approximately 20 mL/minute.

   h) The split delay time is set at approximately 0.75 minutes.

   i) The septum purge is set at approximately 1-2 mL/minute.

2. The following MS parameters are considered recommended starting conditions. The actual conditions and ions used for quantitation will be optimized by the operator and documented in the study file.

   a) Data acquisition is set for selected ion monitoring in electron impact mode at 70 eV.

   b) The solvent delay is set at 4 minutes.

   c) From about 4.0 to 7.0 minutes, the approximate mass to charge ratios (m/z) 241 and 300 (TDG) and m/z 245 and 309 (d_8- TDG) are monitored. The exact ions monitored are determined by running a full scan chromatogram of a high concentration (>15 ug/mL) standard. The dwell time for each ion is set at 10 milliseconds (msec).

J. Analysis of the chromatograms:

1. Calculations:

   a) After injecting the samples, integrate the peaks in the chromatograph. Retention times of approximately 6.0 and 6.1 minutes have been obtained for d_8 –TDG and TDG, respectively, under the previously listed conditions.

   b) The area count is used in analyzing the data. The peak area ratio is calculated by dividing the m/z 241 (TDG) area by the area of the m/z 245 (d_8 –TDG) peak. Alternatively, the peak area ratio may be calculated by dividing the m/z 300 (TDG) area by the area of the m/z 307 (d_8 –TDG) peak. This gives a peak area ratio which when plotted against concentration will yield a positive slope.

   The area counts for the 241,244 m/e peaks are larger than those for the 300,307 m/e peaks, however, there appears to be less interference in the lower concentration levels (<25 ng/mL) when using the 300, 307 m/e values.

K. Calculations:

Generate a regression line for the aqueous/urine standards. Verify that the instrument response is linear using a linear regression model. The values of TDG back calculated using the regression line should vary by no more than 15 - 20% of the spiked level. The value of TDG in the unknown urine sample can now be calculated.

L. Statistical Formula:

The mean, standard deviation and percent relative standard deviation are defined as follows:

$$\text{Mean} = \frac{\sum X_i}{n}$$

$$\text{Standard Deviation} = \text{STD} = \frac{(\sum (X_i - X)^2)^{1/2}}{(n-1)}$$

$$\text{Percent Relative Standard Deviation} = \%RSD = \frac{STD * 100}{X}$$

n = number of results
X = mean experimental result
X_i = experimental result 1 to n

M. Linear Regression Formula:

A linear regression program may be used to generate the slope (a), intercept (b) and correlation coefficient for thiodiglycol (R) in the aqueous and urine samples. If a regression program is not available, calculate the following values:

$$b = \frac{[(\Sigma y)(\Sigma x^2)-(\Sigma x)(\Sigma xy)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$a = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$r = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[(n(\Sigma x^2)-(\Sigma x)^2)^{1/2}(n\Sigma (y^2)-(\Sigma y)^2)^{1/2}]}$$

where,

$y = ax + b$
a = slope
b = y-intercept
r = correlation coefficient
x = amount of analyte in ng
y = ratio of area of analyte to area of internal standard
n = number of replicates

If the over-spike method (H.1.e.) was used to quantitate at low levels (<25 ng/mL TDG) where pre-exposure urine was not available, find the regression line as mentioned above. Set y equal to 0 and solve for x. The absolute value of the x value is the concentration of TDG in the unknown sample (also known as method of standard additions). .

N. <u>References:</u>

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3. U.S. Army Medical Research Institute of Chemical Defense, Aberdeen, Proving Ground, MD, FAX transmission from Dr. Ming Shih, September 28, 1998.

4. Acylation Derivatization Reagents, Pierce Chemical Co., Technical Assistance Copy 0765.

O. <u>Review and Approval:</u>

Originated by: _Janet Ricks_   _4-22-99_
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Reviewed by: _Timothy L Hayes_   _4-22-99_
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APPENDIX D

Package Insert – Test-Mate ChE Kit

EQM Research, Inc.
2585 Montana Ave., Cincinnati, Ohio 45211 USA

Phone: (513) 661-0560
Fax: (513) 661-0567

AChE Erythrocyte Cholinesterase Assay Kit (Model 460)
&
PChE Plasma Cholinesterase Assay Kit (Model 470)

Package Insert

Intended Use

For the quantitative determination of cholinesterase in whole blood to monitor pesticide exposure. For in vitro diagnostic use. For laboratory use by trained laboratory technicians only. The Test-mate ChE Cholinesterase Test System is useful in the assessment of pesticide poisoning. Most organophosphate or carbamate pesticides inhibit the blood enzymes erythrocyte cholinesterase (AChE) and/or plasma cholinesterase (PChE).[1,2] The degree of enzyme inhibition is proportional to the extent of exposure. AChE is generally preferred because of its lower biological variability and lack of interferences relative to PChE. After exposure to pesticides, recovery of AChE activity is usually slower than PChE due to its longer half-life (1 month vs. 2 weeks for PChE).[3,4] Pre-exposure (baseline) measurements of AChE and/or PChE should be obtained to reduce the effect of biological variability.[1]

Principle of the Method

The Test-mate ChE reagents are based on the Ellman method.[5] Acetylthiocholine (AcTC) or butyrylthiocholine (BuTC) is hydrolyzed by AChE or PChE, respectively, producing carboxylic acid and thiocholine which reacts with the Ellman reagent (DTNB, dithionitrobenzoic acid) to form a yellow color which is measured spectrophotometrically at 450nm. The rate of color formation is proportional to the amount of either AChE or PChE.

$$\text{thiocholine ester (AcTC/BuTC)} \xrightarrow{\text{Cholinesterase}} \text{thiocholine}$$

$$\text{thiocholine + DTNB} \xrightarrow{\hspace{2cm}} \text{TNB-thiocholine + TNB (\textit{yellow})}$$
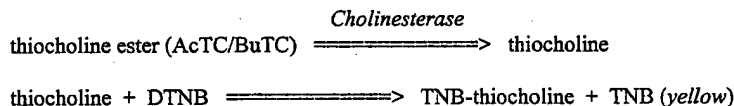
The AChE reagent is >95% specific due to the addition of a specific inhibitor of PChE, As1397 (10-(α-diethylaminopropionyl)-phenothiazine).[6] BuTC is >95% specific for PChE.

Contents

Each assay kit contains three boxes and a package insert. Box one contains 48 assay buffer tubes. Box two contains 48 assay buffer tubes. Box three contains a 96 well reagent plate, 100 capillary tubes (10μL volume), 100 filter papers (capillary wipes), a clear plastic dropper bottle filled with 18mL of distilled water and 2 transfer pipettes. The reagent plate in the AChE Assay Kit has a red "Erythrocyte" label and the PChE Assay Kit has a blue "Plasma" label. The transfer pipettes in the AChE Assay Kit have a red band and the transfer pipettes in the PChE Assay Kit have a blue band. *Note: Never interchange the reagent plate or the transfer pipettes when switching between AChE and PChE testing.*

Instrumentation

The AChE Erythrocyte Cholinesterase Assay Kit and the PChE Plasma Cholinesterase Assay Kit are for use only with the photometric analyzer supplied as part of the Test-mate ChE Cholinesterase Test System and are not intended for use with any other manual or automated test method or equipment.

FOR RESEARCH USE ONLY

GMP CC98

Reagents

1. **Buffer:** 2.0mL per assay tube. Contains phosphate, surfactant, dye and EDTA preservative.
2. **Distilled water:** 18mL in a plastic dropper bottle.
3. **Erythrocyte Cholinesterase Reagent:** (AChE Erythrocyte Cholinesterase Assay Kit) Lyophilized, 96 tests per plate. Store lyophilized reagent at 15 - 30°C, protected from light. Reconstitute with 3 drops of distilled water. Stable 72 hours at 15 - 35°C after reconstitution. Final assay includes: 1mM AcTC, 0.3mM DTNB, 20μM As1397, 50mM potassium phosphate and 0.1% Triton X-100, pH 7.6.

    Plasma Cholinesterase Reagent: (PChE Plasma Cholinesterase Assay Kit) Lyophilized, 96 tests per plate. Store lyophilized regent at 15 - 30°C, protected from light. Reconstitute with 3 drops of distilled water. Stable 72 hours at 15 - 35°C after reconstitution. Final assay includes: 2mM BuTC, 0.3mM DTNB, 50mM potassium phosphate and 0.1% Triton X-100, pH 7.6.

Specimen Collection

Either fresh fingerstick blood or venipuncture blood (anticoagulated with EDTA) can be used. The puncture site should be thoroughly washed before sampling, in order to minimize possible sample contamination from pesticide residue adsorbed to the skin. To avoid clotting, the capillary should be placed into the assay tube within 10 seconds. Cholinesterase can reactivate, especially from carbamate pesticide inhibition during prolonged storage. Such reactivation can produce a "false negative".[7]

Test Procedure

1. Turn on the photometric analyzer. Press the MODE key to select either the AChE assay procedure or the PChE assay procedure. Press the TEST key to begin the assay.
2. Insert the new assay tube into the analyzer. Press the TEST key to continue the assay.
3. When prompted by the analyzer, remove the assay tube. Press the TEST key to continue the assay.
4. Fill the 10μL capillary with blood (wipe excess with filter paper) and place it into the assay tube. Vigorously shake the assay tube for 15 seconds. Align the capillary and then insert the assay tube into the analyzer. Press the TEST key to continue the assay.
5. When prompted by the analyzer, remove the assay tube. Press the TEST key to continue the assay.
6. Dissolve the reagent with 3 drops of distilled water. Add the dissolved reagent to the assay tube using the transfer pipette. Immediately, press the TEST key to continue the assay.
7. Gently shake the assay tube by inversion for 5 seconds. Align the capillary and then insert the assay tube into the analyzer. Press the TEST key to continue the assay.
8. When prompted by analyzer, remove and discard the assay tube. Press the TEST key to continue the assay.
9. Record the analyzer readings, using the TEST key to advance the display. Press the DONE key to finish the assay.

Calibration

The Test-mate ChE photometric analyzer is factory-calibrated. No additional calibration is required.

Quality Control

The use of an unexposed operator is best; the intraindividual variability of both erythrocyte and plasma cholinesterase is less than 5% per week and less than 10% per month.[8] Alternatively, refrigerated venipuncture blood (anticoagulated with EDTA) is stable for at least one month. Controls should be run on each day of testing.

Calculations

The measured cholinesterase activity is calculated by the photometric analyzer using the following equation:

$$U/mL \text{ blood} = \frac{(A/min) \ (mL \text{ assay volume})}{(\varepsilon, \ mM^{-1}cm^{-1}) \ (cm \text{ light path}) \ (mL \text{ blood})}$$

The measured cholinesterase activity is further refined by the following adjustments to derive the final displayed cholinesterase value:

Reagent Blank Adjustment: A small (approximately 15%) nonspecific blank reaction is subtracted from the measured cholinesterase activity.

Temperature Adjustment: Using the temperature sensor in the analyzer, both the measured cholinesterase activity and the reagent blank activity are normalized to 25°C.

Hemoglobin Adjustment: For AChE, hemoglobin normalizes varying sample size and iron status; therefore AChE is most accurately expressed as U/g Hgb.

Limitations

Physiological Interferences: AChE is depressed in paroxysmal nocturnal hemoglobinuria (PNH).[4] In severe macrocytic or microcytic anemia, the ratio of hemoglobin/cholinesterase may interfere with hemoglobin correction and therefore, AChE activity. PChE is depressed in liver failure and malnutrition. PChE is increased in alcoholic/viral hepatitis and infection.[3]

Analytical Interferences: Drugs which inhibit cholinesterase, such as pyridostigmine, will decrease cholinesterase. Pesticide residues adsorbed to the skin can artificially decrease values.[9] Washing the skin with quaternary ammonium-containing detergents, such as benzethonium chloride can also artificially decrease values; check the detergent label before using.

Accuracy

The Test-mate ChE was compared with the Boehringer Mannheim Cholinesterase Kit No. 450035 on the Hitachi 704 (BM/H).[10] The BM/H method is performed on plasma (PChE) or diluted whole blood (AChE) corrected by hematocrit; therefore, in contrast to the Test-mate ChE units of U/mL whole blood at 25°C or U/g Hgb at 25°C, the BM/H results are expressed as U/L plasma (PChE) at 37°C or U/L RBCs (AChE) at 37°C.

Normal Donors: (X,BM/H,Venipuncture) vs. (Y,Test-mate,Fingerstick)

	N	r	Slope	Intercept	Range†
AChE, U/L RBCs vs. U/g Hgb	44	0.78	0.000894	10.8	±25%CV
PChE, U/L plasma vs. U/L blood	44	0.96	0.253	440	±50%CV

Pesticide-Dosed & Normal Donors: (X,BM/H,Venipuncture) vs. (Y,Test-mate,Venipuncture)

	N	r	Slope	Intercept	Range†
AChE, U/L RBCs vs. U/g Hgb	86	0.98	0.00158	0.322	±100%CV
PChE, U/L plasma vs. U/L plasma	87	0.98	0.457	-210	±100%CV

†Note: *r*, the correlation coefficient, is extremely range-sensitive (increases with %CV range).

Precision

Within-run, N=40, 1 - 5U/mL: 3 - 5%CV.   Between-run, N=40, 1 - 5U/mL: 5 - 7%CV.

Linearity

Erythrocyte AChE: 0 - 7U/mL; 0 - 50U/g Hgb.   Plasma PChE: 0 - 7U/mL.

EQM Research insert2b   3

Expected Values

These were determined using normal male and female blood bank donors, between 20 and 60 years of age, located in the midwestern United States.

	N	Mean	SD	Range
AChE, U/mL	40	3.68	0.47	2.77 - 5.57
AChE, U/g Hgb	40	27.1	2.9	21.9 - 37.3
PChE, U/mL	40	2.03	0.40	1.35 - 3.23

Between-Operator Variability

Ten operators each performed ten measurements on both a normal and an abnormal venipuncture sample (N=100). The abnormal sample was prepared by dosing with pesticide (paraoxon).

	Normal				Abnormal			
	AChE U/mL	AChE U/g	PChE U/mL	Hgb g/dL	AChE U/mL	AChE U/g	PChE U/mL	Hgb g/dL
Mean	5.63	33.8	1.72	16.8	1.38	9.7	1.03	14.3
SD	0.21	0.8	0.15	0.5	0.12	0.8	0.08	0.3
%CV	3.7	2.4	8.5	2.7	9.0	7.9	7.5	2.2

Interpretation of Results

Depression of cholinesterase to <50% normal indicates possible pesticide poisoning requiring removal from exposure and/or treatment with anticholinergics such as atropine and pralidoxime.[1] Suspected cases of poisoning can be confirmed by cholinesterase monitoring for a subsequent rise and plateau of activity 1 - 3 months after exposure. If baseline values are obtained, depression of cholinesterase to <70% of baseline can be taken to indicate possible pesticide poisoning.[11]

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APPENDIX E

**Method No. 17/Chemistry
Analytical Method for the Monitoring of Blood Cholinesterase
Activity with the Test-Mate ChE Kit**

ANALYTICAL METHOD FOR THE MONITORING OF BLOOD CHOLINESTERASE ACTIVITY WITH THE TEST-MATE CHE KIT

A. Statement of Work:

The Test-mate ChE cholinesterase test system manufactured by EQM Research, Cincinnati, Ohio is intended for use in the assessment and diagnosis of asymptomatic organophosphate or carbamate poisoning. The test system, using both the erythrocyte and plasma modes will be evaluated for reliability and reproducibility against a previously used method.

B. Purpose:

Most organophosphates (i.e. G-agents, VX) inhibit the blood enzymes erythrocyte acetylcholinesterase (AChE) and/or plasma cholinesterase (PChE). The degree of enzyme inhibition is proportional to the extent of exposure. Determination of AChE is generally preferred because of its lower biological variability and lack of interferences relative to PChE. The Test-mate system is reported to be much less time consuming and labor intensive than previous methods (Technicon, COBAS FARA®). All three methods are based on the Ellman method. Acetylthiocholine (AcTC) or butyrylthiocholine (BuTC) is hydrolyzed by AChE or PChE, respectively, producing carboxylic acid and thiocholine. The thiocholine reacts with Ellman reagent (DTNB, dithionitrobenzoic acid) to form a yellow color which is measured spectrophotometrically at 450 nm. The rate of color formation is proportional to the amount of either AChE or PChE. The AChE reagent is reported to be >95% specific due to the addition of a specific inhibitor of PChE, As 1397 (10-($\propto$-diethylaminopropionyl)-phenothiazine). BuTC is reported to be >95% specific for PChE.

C. Samples:

Either fresh fingerstick blood or venipuncture blood (anticoagulated with EDTA) can be used with this system, however, our testing will be limited to venipuncture blood. Human blood and blood products will be handled as stated in the "Work Instructions for Handling Materials Potentially Contaminated with Blood Borne Pathogens During Sample Analysis". Since blood samples can transmit infectious diseases such as hepatitis and AIDS, exercise universal precautions at all times.

Physiological Interferences: AChE is depressed in paroxysmal nocturnal hemoglobinuria (PHN). In severe macrocytic or microcytic anemia, the ratio of hemoglobin/cholinesterase may interfere with hemoglobin correction and hence AChE activity. PChE is depressed in liver failure and malnutrition and increased in alcoholic/viral hepatitis.

Analytical Interferences: Drugs which inhibit cholinesterase, such as pyridostigmine, will decrease cholinesterase. Pesticide residues, as well as, quaternary ammonium-containing soaps can also decrease values.

D. Materials To Be Used:

The Model 400 Test-mate ChE Cholinesterase Test System contains all the components and reagents necessary for the performance of 96 AChE assays (Model 460 AChE Assay kit). The Model 470 PChE contains only the reagents necessary for 96 PChE assays.

Model 400 Test-mate System:

1. Instruction manual, Rev. F2©1998.

2. Hard shell storage case.

3. Photometric analyzer.

4. Model 460 AChE Assay kit.

5. Reagent opening tool.

6. Assay tube rack.

Model 460 AChE Assay kit:

1. 96 assay buffer vials with black screw caps (2 ml per assay tube; phosphate, surfactant, dye and EDTA preservative).

2. 96 well erythrocyte reagent plate with "Erythrocyte – AChE" label (1 mM AcTC, 0.3 mM DTNB, 20 μM As1397, 50mM potassium phosphate and 0.1 % Triton X-100, pH 7.6).

3. 100 capillary tubes (10 μl volume, Drummond, part number 1-00-0100).

4. 100 filter papers (capillary wipes, Whatman 1, catalog number 1001-042).

5. 30 ml clear plastic dropper bottle filled with 18 ml distilled water.

6. 2 transfer pipets, extended fine tip (similar to VWR 14670-325).
Model 470 PChE Assay kit:

1. 96 assay buffer vials with black screw caps (2 ml per assay tube; phosphate, surfactant, dye and EDTA preservative).

2. 96 well erythrocyte reagent plate with "Plasma -PChE" label (2 mM BuTC, 0.3 mM DTNB, 50 mM potassium phosphate and 0.1% Triton X-100, pH 7.6).

3. 100 capillary tubes (10 µl volume, Drummond, part number 1-00-0100).

4. 100 filter papers (capillary wipes, Whatman 1, catalog number 1001-042).

5. 30 ml clear plastic dropper bottle filled with 18 ml distilled water.

6. 2 transfer pipets, extended fine tip (similar to VWR 14670-325).

E. **Equipment Not Included in Kits:**   *Note: Equivalent equipment may be substituted when necessary*

1. Vacutainer tubes, 7 ml capacity (purple top with EDTA).

2. Blood, may be stored for up to one month in vacutainer tubes, at 1-10 °C.

3. Volumetric glassware (Class A, various sizes).

4. Volumetric pipets (Class A, various sizes).

5. Miscellaneous glassware.

6. Syringes, 10, 50, 100 µl.

7. GD, neat.

8. Polypropylene microcentrifuge tubes, 2.0 ml.

9. Pipettor, variable or fixed volume, positive displacement.

10. Pipettor, variable or fixed volume, air displacement

11. Isotonic saline (0.9 % in deionized water), 250 ml.

12. Disposable pipets, glass or plastic.

13. Centrifuge, Eppendorf 5414 , IEC Centra-8R or equivalent.

F. Methodology for Blood Collection:

All blood draws will be done by a Battelle registered nurse, doctor or certified phlebotomist. Donors will be volunteers from the MREF staff. All volunteers will be given a unique identification number known only to the individual and those working on the task. No record of the donors names will be kept in the permanent study file. All other conditions as listed in the Human Subjects Research Questionnaire dated June 19,1998 will be adhered to. Donors will be required to sign the Human Blood Donor Consent Form, Form No. MREF-Chemistry-41 before venipuncture can proceed.

Fill six – seven ml venipuncture tubes with the blood from each of seven volunteers. Submit one tube from each individual to the appropriate MREF personnel for analysis of both AChE and PChE cholinesterase (in duplicate) by the traditional COBAS FARA® method. Label each tube with the donor identification number, date and time the blood was drawn and store the remaining vials between 1-10 °C when analysis is not in progress.

G. Outline of Test-mate Test Procedure:

1. Turn on the photometric analyzer. Press the **MODE** key to select either the AChE assay procedure or the PChE assay procedure. Press the **TEST** key to begin the assay.

2. Insert a new assay vial into the analyzer. Press the **TEST** key to continue the assay. The analyzer will show *"Blanking"* on the screen and a time from 10-1 seconds.

3. When prompted by the analyzer *("Remove tube")*, remove the assay vial. Press the **TEST** key to continue the assay.

4. Fill the 10 µl capillary with blood. Wipe the excess blood off of the outside of the capillary by placing it on top of a piece of the filter paper and gently rolling the capillary over the filter paper. Place the blood filled capillary into the assay vial. Shake the assay vial vigorously for approximately 15 seconds or until all of the blood has been removed from the capillary into the buffer solution in the vial.

5. Align the capillary in the vial with the black line on the analyzer and then insert the assay vial into the analyzer. Press the **TEST** key to continue the assay.

6. When prompted by the analyzer *("Remove tube")*, remove the assay vial. Press the **TEST** key to continue the assay.

7. Remove the plastic cover from one well in the 96 well plate using the reagent opening tool. Dissolve the reagent in the well with 3 drops of distilled water. Add the dissolved

reagent to the assay vial using the transfer pipette. Immediately, press the **TEST** key to continue the assay.

8. Shake the assay vial by gentle inversion for approximately 5 seconds. Align the capillary in the vial with the black line on the analyzer and then insert the assay vial into the analyzer. Press the **TEST** key to continue the assay.

9. When prompted by analyzer *("Remove tube")*, remove and discard the assay vial. Press the **TEST** key to continue the assay.

10. Record the analyzer readings using the **TEST** key to advance the display from one reading to the next. Press the **DONE** key to finish the assay. NOTE: Pressing the **DONE** key will erase all previously obtained readings. Values given in AChE mode are:

AChE	(U/ml)	% N (% of normal)
Hgb	(g/dl)	% N (% of normal)
Q	(U/g)	% N (% of normal)

Q is the hemoglobin corrected value of erthrocycte cholinesterase. It is computed by dividing the AChE result by the Hgb result.

Values given in PChE mode are:

PChE	(U/ml)	% N (% of normal)
Hgb	(g/dl)	% N (% of normal)

NOTE: REFER TO THE OPERATOR'S MANUAL FOR PICTURES AND FURTHER CLARIFICATION OF EACH STEP

H. Analytical Procedure:

1. Precision and Operator Variability:

   a. Allow the blood in one or two vacutainer tube from each volunteer to reach room temperature.

   b. Slowly invert each tube approximately 20 times or until there are no red blood cells remaining on the bottom of the tube.

c. Analyze each of the seven samples for both AChE and PChE using the Test-mate kit, in duplicate, and record the values as stated in G.10. in the laboratory notebook.

d. Repeat step c, with the analyses being performed by a second analyst.

e. Repeat steps c and d on four more non-consecutive days. Both analysts must remain the same throughout the five day testing.

2. Linearity:

a. Randomly choose the blood of five different donors from the blood already drawn. Allow two or three vacutainer tubes (depending on volume in tube) from each donor to come to room temperature.

b. Slowly invert the vacutainer tubes approximately 20 times or until there are no red blood cells remaining on the bottom of the tubes.

c. For each of the five different blood samples, aliquot 1ml of blood into 6 – 2 ml polypropylene microcentrifuge tubes (30 tubes total). This aliquoting may be done with a positive or air displacement, fixed or variable volume pipettor. Ensure that the tubes are labeled with the donor identification number and quantity of packed red blood cells added (see step k).

d. Using a syringe, spike one full (5-10 ml) vacutainer tube of blood from each donor with approximately 1 µl of neat GD. Follow all procedures as listed in Battelle SOPs MREF. I-002 and MREF. I-003 for Handling of CSM and XCSM. The blood tubes will be placed in the hood at least 2 feet from the CSM vial. The time that the blood tubes are in the CSM hood will be minimized. The doser will not touch the outside of the tubes with gloves or the agent syringe.

e. An individual wearing clean gloves will slowly invert each of the 5 tubes of blood approximately 20 times before removal from the CSM hood and handling as XCSM. Centrifuge to reconcentrate the red blood cells on the bottom of the tube.

f. Using a disposable pipet, remove the plasma from each tube and place it in a decon bucket containing 5 % bleach.

g. Add 3 mL of isotonic saline to each tube, slowly invert 20 times or until there are no red blood cells on the bottom of the tube and again centrifuge.

h. Discard the saline into the same decon bucket as in step f.

i. Repeat steps g and h four more times.

j. At this step, the contents will not be considered XCSM. Centrifuge the microcentrifuge tubes in step c. to concentrate red blood cells on the bottom of the tubes.

k. Using a positive displacement pipettor, remove 0 μL, 10 μL, 20 μL, 30 μL, 40 μL and 50 μL of packed red blood cells from the microcentrifuge tubes in step c.

l. Using a positive displacement pipettor, add 10 μL, 20 μL, 30 μL, 40 μL and 50 μL of the GD treated packed red blood cells to each of the appropriate polypropylene microcentrifuge tubes from step c.

m. Repeat step j. with the other four donor blood samples.

n. Analyze all microcentrifuge tubes (30) for AChE and PChE using the Test-mate kit procedure as outlined in Section G. Record all values in the laboratory notebook.

I. Results and Calculations:

The expected values for AChE and PChE, as determined from the manufacturer's study of 40 blood bank donors, are listed on page 16 of Test-mate kit operator's manual. Also included are the calculated mean, standard deviation and range for these test values, where:

$$\text{Mean} = \overline{X} = \frac{\sum X_i}{n}$$

$$\text{Standard Deviation} = \frac{(\sum (X_i - \overline{X})^2)^{1/2}}{(n-1)}$$

n = number of results
X = experimental result
i = experimental result 1 to n

Using the results determined in section H.1.c-e, calculate and list in tabular form the mean, standard deviation and range of AChE and PChE as determined by each of the two analysts on each of the five days. Also include in the table the results obtained by the manufacturer as listed on page 16 and 18 of the operator's manual.

Using the results obtained in section H.2., analyze the data with the linear regression model in the form of y = ax + b. Using a linear regression program, generate the slope (a), intercept (b) and correlation coefficient for AChE and PChE for each of the five donor samples. If a regression program is not available, calculate the following values:

$$b = \frac{[(\Sigma y)(\Sigma x^2)-(\Sigma x)(\Sigma xy)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$a = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[n(\Sigma x^2)-(\Sigma x)^2]}$$

$$r = \frac{[n(\Sigma xy)-(\Sigma x)(\Sigma y)]}{[(n(\Sigma x^2)-(\Sigma x)^2)^{1/2}(n\Sigma (y^2)-(\Sigma y)^2)^{1/2}]}$$

where,

$y = ax + b$
a = slope
b = y-intercept
r = correlation coefficient
x = amount of GD in μl
y = value of AChE or PChE
n = number of replicates

The value of r should be ≥ 0.99 for an acceptable correlation coefficient of the regression line. Compare in tabular form, the regression data with that obtained by the manufacturer as listed on page 17 of the operator's manual.

J. References

1. Test-mate ChE Cholinesterase Test System (Model 400) Instruction Manual, EQM Research, Inc., 2585 Montana Avenue, Cincinnati, Ohio 45211. Rev.F2 ©1998.

2. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961; 7-88-95.

3. MREF Method No. 33/In Vitro.

4. EQM Research, Inc., FDA 510(k) premarket notification, Sections 14, 15, 16, 17 and 18, Oct 28, 1996.

5. Battelle SOP MREF. I-002, Storage, Dilution, and Transfer of GA, GB, GD, GF, TGD, VX, HD, HL, HN and L When CSM Concentration/Quantity is Greater than Exempt Levels.

6. Battelle SOP MREF. I-003, Receipt, Transfer, Storage, and Use of Exempt Chemical Surety Materiel (XCSM).

7. MREF Training Document - Work Instructions for Handling Materials Potentially Contaminated with Blood Borne Pathogens During Sample Analysis.

K. Review and Approval

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Senior Quality Assurance Specialist

APPENDIX F

Method No. 19/Chemistry
Method for Verification of Nerve Agent Exposure

METHOD FOR VERIFICATION OF NERVE AGENT EXPOSURE – MEASURING ALKYLMETHYLPHOSPHONIC ACIDS IN URINE BY GAS CHROMATOGRAPH/MASS SPECTROMETER

A. <u>Statement of Work:</u> This method describes the detection of the metabolites of three toxic organophosphorus compounds (sarin, soman, and GF) after derivatization with pentafluorobenzyl bromide (PFBBr) by gas chromatography (GC) coupled with mass spectrometry (MS). Battelle has documented the calibration range from 5 ng/mL to 200 ng/mL of isopropyl methylphosphonic acid (IMPA), pinacolyl methylphosphonic acid (PMPA) or cyclohexyl methylphosphonic acid (CMPA). The lowest detectable concentrations are matrix dependent but range from approximately 0.3 – 0.5 ng/mL with quantitation limits approximately 20 ng/mL (CV 15%) for all three phosphonic acids. Tri-deuterated isopropyl methylphosphonic acid (d_3-IMPA) and tri-deuterated pinacolyl methylphosphonic acid (d_3-PMPA) are used as internal standards. Through the use of spiked urine standards and the internal standards, a linear regression plot is developed to determine the phosphonic acid concentrations.

B. <u>Purpose:</u> In animals (including human beings) exposed to the toxic organophosphorus nerve agents, substantial amounts of the parent compounds are hydrolyzed to their corresponding phosphonic acids and the rest are covalently bound to enzymes and tissue proteins. Urinary excretion of the metabolite is the primary elimination route for these three compounds, which can therefore be used to confirm an exposure.[1]

C. <u>Samples:</u> The method has been validated for use in both aqueous and human urine samples. Ideally, standards should be prepared in the pre-exposure urine of the person involved; however, this is not feasible in most situations. During the validation of this method, it was shown that the differences in reproducibility between various aqueous and urine matrices are measurable but small. Since most analyses will contain urine from several different patients, standards are prepared in high purity water.

If the submitted urine samples are from other species, the method should be re-validated before analysis.

D. <u>Materials: (equivalent items may be substituted)</u>

   1. Pinacolyl methylphosphonic acid-d_3 (Material #: 98R-001) [CAS 172023-63-1], cyclohexyl methylphosphonic acid (Material #: 98R-002) [CAS 1932-60-1], isopropyl methylphosphonic acid (Material #: 98R-004) [CAS 1832-54-8] and isopropyl methylphosphonic acid-d_3 (Material #: 98R-005)[CAS 172023-64-2] were prepared by Dr. Allison Fentiman, Battelle Memorial Institute, 505 King Ave., Columbus, Ohio 43201.

2. Pinacolyl methylphosphonic acid, Aldrich Chemical Co., catalog number 38,658-8, [CAS 616-52-4].

3. Concentrated hydrochloric acid, ACS grade, Sigma Chemical Co., catalog number H-7020, [CAS 7647-01-0].

4. Powdered anhydrous potassium carbonate, Aldrich Chemical Co., catalog number 34,782-5, [CAS 584-08-7].

5. Sodium chloride, J. T. Baker, catalog number 4058-01, [CAS 7647-14-5].

6. Methanol, HPLC grade, Burdick and Jackson Labs, catalog number BJ230-4, [CAS 67-56-1].

7. Methylene chloride, Burdick and Jackson Labs, catalog number GC299-4, [CAS 75-09-2].

8. 18-crown-6 ether, Aldrich Chemical Co., catalog number 18,665-1, [CAS 17455-13-9].

9. Pentafluorobenzyl bromide (PFBBr), Pierce, catalog number 58220, [CAS 1765-40-8].

10. Carbon tetrachloride, Sigma Chemical Co., catalog number C5331, [56-23-5].

11. Water, Baker Instra Analyzed, VWR, catalog number JT4219-3, [7732-18-5].

E. Equipment: (equivalent items may be substituted)

1. Nitrogen and helium gases, 99.9% pure.

2. GC/MS, with an electron impact source.

3. Polypropylene microcentrifuge tubes (conical with attached tops), approximately 2 mL, VWR, catalog number 20170-610.

4. Polypropylene microcentrifuge tubes (with screw caps), approximately 2 mL, Corning, catalog number 25758-2.

5. Reacti-Therm III (Pierce, Rockford, IL, 18935) an evaporator equipped with aluminum blocks (Reacti-Block,V-1) and gas nozzles (Reacti-Vap III, 18785) .

6. Centrifuge, Eppendorf Centrifuge 5414.

7. Vortex mixer, VWR, Vortexer 2.

8. DB-5MS bonded-phase capillary column (Alltech Assoc., Deerfield, IL, 93912), 25 meters, 0.2-mm inner diameter, 0.33-μm film thickness.

9. Pipettors and tips, air displacement, ranges 10-100 μL, 20-200 μL, and 100-1000 μL.

10. Glass scintillation vials (20 mL capacity), VWR, catalog number 66021-726.

11. Teflon™ cap liners for glass scintillation vials, Thomas Scientific, catalog number 2390J76.

12. pH paper, pHydrion, Microfine 0.8-2.0 pH.

13. Inserts for autosampler vials, approximately 0.2 mL, Hewlett Packard, catalog number 5181-1270.

14. Volumetric flasks, 10 and 25 mL capacity, Class A.

15. Pipets, 1, 5, 10 mL, Class A.

16. Glass syringes: 10 μL, 25 μL, 50 μL, 250 μL, 500 μL, 1000 μL, and 1.5 mL capacity.

17. 100-mg BondElut, C18 cartridges, Varian/Rainin, catalog number R012102001.

18. 5-mL disposable syringe with adapter to fit BondElut cartridges.

F. Methodology For Urine Collection: The method of urine collection will be defined in the protocol; however, it is imperative that the samples are well mixed and representative.

Store the urine samples in plastic containers, labeled with the source and/or laboratory record book number and date. Freeze the samples until needed for analysis. It is advantageous to divide a single sample between smaller containers with aliquots defrosted as needed. This will limit the number of freeze/thaw cycles necessary. Expiration date of the urine samples will be one year from the date of collection.

G. Preparation of the standards: (record all standards in a numbered laboratory record book, equivalent dilutions may be made)

1. Preparation of the stock solutions:

a. *5 mg/mL Stock IMPA Standard.* Into a 10-mL volumetric flask weigh out approximately 50 ± 5 milligrams (mg) of IMPA, dilute to volume with Instra Analyzed water and vortex to mix. Transfer this solution to a glass scintillation vial and label with the solution identification (*Stock IMPA Standard*), the concentration in mg/mL, the preparation and expiration dates, laboratory record book number and page and the name of the preparer. The stock solution is stable for at least 3 months if kept refrigerated[1].

b. *5 mg/mL Stock PMPA Standard.* Into a 10-mL volumetric flask weigh out approximately 50 ± 5 milligrams (mg) of PMPA, dilute to volume with Instra Analyzed water and vortex to mix. Transfer this solution to a glass scintillation vial and label with the solution identification (*Stock PMPA Standard*), the concentration in mg/mL, the preparation and expiration dates, laboratory record book number and page and the name of the preparer. The stock solution is stable for at least 3 months if kept refrigerated[1].

c. *5 mg/mL Stock CMPA Standard.* Into a 10-mL volumetric flask weigh out approximately 50 ± 5 milligrams (mg) of CMPA, dilute to volume with Instra Analyzed water and vortex to mix. Transfer this solution to a glass scintillation vial and label with the solution identification (*Stock CMPA Standard*), the concentration in mg/mL, the preparation and expiration dates, laboratory record book number and page and the name of the preparer. The stock solution is stable for at least 3 months if kept refrigerated[1].

d. *5 mg/mL Stock d₃-IMPA Standard.* Into a 10-mL volumetric flask weigh out approximately 50 ± 5 milligrams (mg) of d_3-IMPA, dilute to volume with Instra Analyzed water and vortex to mix. Transfer this solution to a glass scintillation vial and label with the solution identification (*Stock d_3-IMPA Standard*), the concentration in mg/mL, the preparation and expiration dates, laboratory record book number and page, and the name of the preparer. The stock solution is stable for at least 3 months if kept refrigerated[1].

e. *5 mg/mL Stock d₃-PMPA Standard.* Into a 10-mL volumetric flask weigh out approximately 50 ± 5 milligrams (mg) of d_3-PMPA, dilute to volume with Instra Analyzed water and vortex to mix. Transfer this solution to a glass scintillation vial and label with the solution identification (*Stock d_3-PMPA Standard*), the concentration in mg/mL, the preparation and expiration dates, laboratory record book number and page and the name of the preparer. The stock solution is stable for at least 3 months if kept refrigerated[1].

f. *3 mg/mL 18-crown-6 ether in Methylene Chloride.* Weigh 150 ± 5 mg of 18-crown-6 ether into a 50-mL volumetric flask. Dilute to volume with methylene chloride, vortex and transfer into a well-sealed bottle or vial equipped with Teflon™ liner. If kept sealed and refrigerated, this solution may be stored for approximately 3 months[1].

2. Preparation of the working standards:

a. *5 μg/mL IMPA, PMPA, CMPA Mixed Working Standard.* Using a syringe, transfer 25 μL <u>each</u> of the *5 mg/mL Stock IMPA, PMPA and CMPA Standards* into a 25-mL volumetric flask. Dilute to volume with Instra Analyzed water and vortex to mix. Transfer the solution to a glass scintillation vial and label with the solution identification (*5 μg/mL IMPA, PMPA, CMPA Working Standard*), the preparation and expiration dates, laboratory record book number and page, and the name of the preparer. This solution is stable for at least 3 months if kept refrigerated[1].

b. *5 μg/mL d₃-PMPA and d₃-IMPA Mixed Working Standard.* Using a syringe, transfer 25 μL <u>each</u> of the *5 mg/mL d₃-PMPA and d₃-IMPA Stock Standards* into a 25-mL volumetric flask. Dilute to volume with Instra Analyzed water and vortex to mix. Transfer the solution to a glass scintillation vial and label with the solution identification (*5 μg/mL d₃-PMPA and d₃-IMPA Working Standard*), the preparation and expiration dates, laboratory record book number and page, and the name of the preparer. This solution is stable for at least 3 months if kept refrigerated[1].

c. *1 μg/mL IMPA, PMPA, CMPA Mixed Working Standard.* Using a syringe, transfer 2 mL of the *5 μg/mL IMPA, PMPA, CMPA Mixed Working Standard* into a 10-mL volumetric flask. Dilute to volume with Instra Analyzed water and vortex to mix. Transfer the solution to a glass scintillation vial and label with the solution identification *(1 μg/mL IMPA, PMPA, CMPA Working Standard)*, the preparation and expiration dates, laboratory record book number and page, and the name of the preparer. This solution is stable for at least 3 months if kept refrigerated[1].

H. <u>Analytical Method</u>:

1. Preparation of sample/standard microcentrifuge tubes:

a. A minimum of three standard concentration levels plus a blank are necessary for the analysis, five levels are recommended. In all cases, the concentrations of the standards must bracket the concentrations of the actual samples. Label the microcentrifuge tubes that are to be used to contain the standards (i.e., 0, 5, 10, 20, 40, 100, 125, 175, and 200 ng/mL). Into each of the tubes pipet 1.0 mL of Instra Analyzed water.

b. Using the appropriate size syringe, add 0, 5, 10, 20, 40, 100, 125, 175, or 200 μL of the *1 μg/mL IMPA, PMPA, CMPA Mixed Working Standard* to tubes labeled 0, 5, 10, 20, 40, 100, 125, 175, or 200 ng/mL, respectively.

c. Using the appropriate size syringe, add 20 μL of the *5 μg/mL d_3-PMPA and d_3-IMPA Mixed Working Standard* to each of the microcentrifuge tubes. Vortex each tube.

d. For urine overspike samples; repeat steps H.1.a to c. using urine in place of the water in step a.

e. For the analysis of the urine of actual nerve agent exposed victims/laboratory animals, add 1.0 mL of the urine to microcentrifuge tubes as in step a. Do not spike with IMPA, PMPA or CMPA. Continue with step H.1.c. It is recommended that these urine samples be analyzed in triplicate.

f. Add 3-4 drops of concentrated HCl to each of the microcentrifuge tubes and vortex. Check the pH of the solution in each tube. To accomplish this, open the cap of each tube and touch the tip of a glass disposable pipet to the inside top lid of the tube in order to remove a drop of liquid. Place the drop on pH paper calibrated for the range of 0.8-2 pH units. Use a clean pipet for each sample. The pH in each tube should be less than 1. If not, add increments of HCl or NaOH dropwise and repeat vortexing and sampling until the pH of each tube is less than 1.

2. Sample Extraction:

a. Prepare one C18 solid-phase cartridge for each of the microcentrifuge tubes. This is done by passing 2 – 1-mL aliquots of methanol followed by 2 – 1-mL aliquots of Instra Analyzed water through each cartridge. Do not allow the cartridges to go dry between the additions of water or methanol. Discard the washings.

b. Add the solution in each centrifuge tube to its corresponding solid-phase cartridge. Discard the solution that passes through the cartridge, since the analytes of interest are adsorbed onto the C18 in the cartridge.

c. Using a 5-mL plastic syringe with adapter, slowly force air through each C18 cartridge until no more liquid can be seen exiting from the bottom of the cartridge. Using the same syringe and adapter slowly pass an additional 2 – 5-mL volumes of air through each cartridge.

d. Rinse each cartridge with 1 mL of 20% (w/v) sodium chloride in 0.1 N HCl. Discard the washings.

e. Using a 5-mL plastic syringe with adapter, again force air through each C18 cartridge until no more liquid can be seen exiting from the bottom of the cartridge. Using the same syringe and adapter slowly pass an additional 2 –5-mL volumes of air through each cartridge.

f. Using 1 mL of methanol, elute the analytes of interest from the extraction cartridge into a new set of labeled microcentrifuge tubes (with screw caps) containing 20 ± 2 mg powdered anhydrous potassium carbonate. (NOTE: the potassium carbonate must be heated to 130°C in a drying oven, at a minimum of overnight, before use. As a matter of convenience, store the potassium carbonate in the oven when not in use.)

g. Using the same procedure as in H.2.c. and e., force air through the cartridge to transfer any remaining methanol into the microcentrifuge tube.

h. Evaporate the methanol in each tube to dryness under a stream of N_2 in the Reacti-Therm III set between 65°C and 70°C. This normally takes approximately 20-25 minutes to accomplish.

i. Using syringes, add 1 mL of methylene chloride containing 18-crown-6 ether and 10 μL of PFBBr to the residue in each tube. Vortex and heat each capped tube in the Reacti-Therm III for 1 hour at 45°C.

j. Vortex each tube every 15 minutes of the 1-hour heating period.

k. At the end of the heating step, cool each tube to room temperature and using a syringe transfer the methylene chloride solutions to clean conical bottom microcentrifuge tubes.

l. Evaporate the liquid in each tube to dryness in the Reacti-Therm III set to room temperature (20 – 30°C) under a stream of N_2.

m. Using syringes, add 100 μL of carbon tetrachloride and 100 μL Instra Analyzed water to the residue in each tube.

n. Briefly vortex each tube and centrifuge for 1 minute.

o. Using a syringe, transfer the organic layer (lower carbon tetrachloride layer) into 2-mL autosampler vials equipped with 0.2-mL inserts.

p. Inject 1 to 2 μL of the sample into the GC/MS.

I. GC/MS parameters:

1. The following GC parameters are considered recommended starting conditions. The actual conditions will be optimized by the operator.

   a. Column - DB-5MS bonded-phase capillary column (Alltech Assoc., Deerfield, IL, 93912), 25 meters, 0.2-mm inner diameter, 0.33-μm film thickness.

   b. The injector port temperature is 200°C.

   c. The source temperature is 225°C.

   d. The oven temperature is programmed as follows: initial temperature is held 45°C for 1 minute, programmed from 45-190°C at a rate of 20°C/minute, held at 190°C for 5 minutes, then programmed from 190 to 320°C at 20°C/minute, and finally held at 320°C for 2 minutes.

   e. The linear velocity with helium is set at 35 cm/sec at 100°C using freon.

   f. The split flow is at 50 mL/minute.

   g. The septum purge is set at approximately 2 mL/minute.

   h. The split delay time is set at 1.0 minutes.

   i. The solvent delay is set to 9.0 minutes.

2. The following MS parameters are considered recommended starting conditions. The actual conditions and ions used for quantitation will be optimized by the operator.

   a. Data acquisition is set for selected ion monitoring in electron impact mode at 70 eV.

   b. The dwell time for each ion is set at 40 milliseconds (msec).

   c. Fragment ions characteristic of the derivatized compounds of interest are monitored (303 and 256 for IMPA and PMPA; 306 and 259 for d_3- IMPA, and d_3- PMPA; 181, 256 and 277 for CMPA). Quantitation is accomplished using 256 m/e for the derivatized analytes while 259 m/e is used for the derivatized deuterated analogs.

J. <u>Analysis of the Chromatograms:</u> PMPA is composed of four stereoisomers due to the asymmetric centers at the phosphorus atom and the pinacolyl carbon. Under the chromatographic conditions employed, the stereoisomers are separated into two diastereomer pairs. Quantitation in the original method[1] is performed by combining the integrated peak area for each peak. Since the software for our GC/MS will not sum these peak areas, our results will be reported listing the individual diastereomer pairs. Either method is acceptable. Calibration lines are constructed by plotting the ratio of the peak area of the fragment ion at m/z 256 to the peak area of the corresponding m/z 259 fragment ion of the deuterated internal standard against standard analyte concentration. The second d_3-PMPA peak is used for the internal standard for CMPA.

K. <u>Calculations:</u> Generate a regression line for the aqueous standards. Verify that the instrument response is linear using a linear regression model. The values of IMPA, PMPA and CMPA back calculated using the regression line should vary by no more than 15% of the spiked level. The value of each phosphonic acid in the unknown urine sample can now be calculated.

L. <u>Statistical Formulae:</u> The mean, standard deviation and percent relative standard deviation are defined as follows:

$$\text{Mean} = \frac{\sum X_i}{n}$$

$$\text{Standard Deviation} = \text{STD} = \frac{(\sum (X_i - X)^2)^{1/2}}{(n-1)}$$

$$\text{Percent Relative Standard Deviation} = \%\text{RSD} = \frac{\text{STD} * 100}{\text{Mean}}$$

where, n = number of results
X = experimental result
i = experimental result 1 to n

M. <u>Linear Regression Formulae:</u> A linear regression program may be used to generate the slope (a), intercept (b) and correlation coefficient for IMPA, PMPA, and CMPA (R) in the aqueous and urine samples. If a regression program is not available, calculate the following values:

$$b = \frac{[(\sum y)(\sum x^2) - (\sum x)(\sum xy)]}{[n(\sum x^2) - (\sum x)^2]}$$

$$a = \frac{[n(\sum xy)-(\sum x)(\sum y)]}{[n(\sum x^2)-(\sum x)^2]}$$

...

$$r = \frac{[n(\sum xy)-(\sum x)(\sum y)]}{[(n(\sum x^2)-(\sum x)^2)^{1/2}(n\sum(y^2)-(\sum y)^2)^{1/2}]}$$

$$y = ax + b$$

where,   a = slope
          b = y-intercept
          r = correlation coefficient
          x = amount of analyte in ng
          y = ratio of area of analyte to area of internal standard
          n = number of replicates

N. References:

1. Technical Bulletin, TB MED 296, May 1996; Assay Techniques for Detection of Exposure to Sulfur Mustard, Cholinesterase Inhibitors, Sarin, Soman, GF and Cyanide; Chapter 4, Verification of Nerve Agent Exposure-Measuring Alkylmethylphosphonic Acids in Urine By Gas Chromatograph/Mass Spectrometer.

2. Assay Technique for Detection of Exposure to Soman (from TB MED 296) Preparation of Standards and Samples for Analysis of Pinacolyl methylphosphonic acid in Urine; USAMRICD; November, 1998.

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